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OBSERVATIONS ON THE NUTRITIONAL REQUIREMENTS OF BACILLUS COAGULANS[1]

T. W. HUMPHREYS[2] AND R. N. COSTILOW

Abstract

The nutritional requirements of 14 cultures of *Bacillus coagulans*, which were isolated and identified in this laboratory, and eight other authentic strains of this organism, were studied at 37° C. Biotin and thiamine were required by all strains in a semisynthetic medium containing enzymatic casein hydrolyzate, glucose, and mineral salts. In addition, one strain required niacin. An additional requirement for folic acid (or PABA) was noted for most strains in a synthetic medium. With few exceptions, the amino acid requirements were non-specific. Glutamic acid appeared essential for a few strains and stimulated others.

Introduction

Many conflicting reports have appeared in the literature on the nutritional requirements of *Bacillus coagulans*, the organism considered to be the chief cause of flat-sour spoilage of tomato juice. Andersen and Werkman (2) found that *Bacillus dextrolacticus* (*Bacillus coagulans* (13)) grew in a basal medium containing either thiamine or riboflavin plus an unidentified ether-soluble factor obtained from acidified yeast extract. The yeast factor could be replaced by three amino acids: viz., glutamic acid, cystine, and either arginine or threonine. A single strain of *B. coagulans* studied by Cleverdon *et al.* (8) required biotin, thiamine, and niacin at 37° and 55° C. in a casein digest basal medium supplemented with L-cystine and DL-tryptophan. Growth and sporulation were better at the lower temperature. Allen (1) found that three strains of *B. coagulans* grew in a simple basal medium containing glucose and monosodium glutamate. The addition of casein hydrolyzate permitted an additional two strains to grow, while another two strains required a mixture of carbon sources and a richer mineral complement. In a more comprehensive

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Contribution No. 433 from the Bacteriology Division, Science Service, Department of Agriculture, Ottawa, Canada, and the Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan, U.S.A. This work forms part of a thesis submitted by the senior author to Michigan State University in November, 1956, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Michigan Agricultural Experiment Station, Journal Article No. 2006.
[2]Product Development, Chemical Division, Merck & Co., Inc., Rahway, N.J.

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study, Knight and Proom (11) found that the nitrogen requirements of 15 new isolates of *B. coagulans*, along with a reference strain of this organism (ATCC 7050), were satisfied by casein digest but not by ammonia. Although thiamine and biotin were usually required, some strains, or selected variants thereof, became self-sufficient with respect to one or the other of these vitamins upon subculture. In further observations, Proom and Knight (12) found this group to be nutritionally homogeneous with respect to their requirements for biotin and thiamine in a 14-amino acid medium. The amino acid demands were considered complex in the light of the absence of growth in a 7-amino acid medium. The authors could not explain differences in their results from those of Cleverdon *et al.* (8) and of Allen (1). Baker *et al.* (4) mentioned that a strain of *B. coagulans* grew in a basal medium supplemented with either glycerol or carbohydrate. Later, Baker *et al.* (3) found that a strain of this organism required folic acid and recommended it for the assay of this vitamin. Campbell and Williams (6) noted that the vitamin and amino acid requirements of four strains of *B. coagulans* varied with the temperature of incubation. Folic acid was required by all strains at 45° and 55° C. but not necessarily at 36°. In another publication (7), they reported on variation with respect to the biotin requirement of six strains of this organism. Bhat and Bilimoria (5) noted that *B. coagulans* failed to grow in a medium containing inorganic nitrogen at 37° or 55° C. in the presence or absence of biotin. Two strains required sulphur-containing amino acids at 55° C. and one strain required the presence of thiamine and histidine. Neither strain required these supplements at 37° C.

Although reports in the literature indicate that the vitamin and amino acid requirements of *B. coagulans* may not be numerous, there remains considerable confusion. Different workers tested different strains using various methods and incubation temperatures. Most investigations have involved too few strains to provide much information on variation within the species. Consequently, the present investigation was undertaken in the hope that the examination of a variety of strains under similar conditions would provide a more complete understanding of the species and perhaps shed light on the conflicting evidence found in the literature.

Methods

Glassware

Pipettes were cleaned by being soaked overnight in potassium dichromate cleaning solution, rinsed in tap water in an automatic pipette washer for 1 day, rinsed thoroughly in distilled water, autoclaved in distilled water, and dried in a hot-air oven. In the earlier work, glassware was cleaned in hot sulphuric acid containing potassium nitrate, rinsed in tap water until neutral to bromthymol blue, then rinsed in distilled water, and dried in the same manner as were the pipettes. In later work, test tubes were cleaned with Alconox* detergent in a manner similar to that described by Campbell and Williams (6).

*Manufactured by Alconox Inc., Jersey City, N.J.

Cultures

The identity of each culture was confirmed by the methods of Smith *et al.* (13). On this basis, 15 strains of *B. coagulans* were selected from a group of sporeforming bacteria freshly isolated by Johns (9) from the wash waters of canning plants that pack tomato juice. The following cultures of *B. coagulans* were also included: NRC strain B-36; ATCC strains 7050 and 10545; ATCC strain 12245, which was reported to require folic acid (3); strains C2253, 43P, and 711 obtained from Dr. C. S. Pederson, New York State Experimental Farm, Geneva, N.Y.; strain 1604 supplied by Dr. A. H. Jones of this laboratory, who isolated it from commercial tomato juice showing evidence of flat-sour spoilage. Stock cultures were carried on nutrient agar (Difco) plus 0.25% yeast extract and 0.1% dextrose.

Inocula

Cells for inocula were obtained from 20–24 hour liquid cultures. In determining the vitamin requirements in semisynthetic media, cells were harvested by centrifugation, washed three times in distilled water, and resuspended in distilled water to give a final light transmittance of 75% at 545 mμ wave length in a Luxtrol photoelectric colorimeter. Cells washed in *M*/20 phosphate buffer were employed during studies in synthetic media. A volume of 0.05 ml. was used to inoculate test media. An incubation temperature of 37° C. was employed throughout. Growth was recorded as percentage transmittance after 72 hours' incubation, using a Luxtrol photoelectric colorimeter at 545 mμ wave length.

Media

In preliminary studies, vitamin-free enzymatic casein hydrolyzate (Nutritional Biochemicals Corp.) proved superior to casamino acids (Difco) as a nitrogen source in semisynthetic media containing known vitamins in place of yeast extract (Difco). Results with casamino acids were inconsistent. Various attempts to improve results with supplements of L-cystine and DL-tryptophan, yeast extract ash, calcium chloride, and sodium acetate were not successful. Based on these studies, a basal medium was devised for the preparation of inocula and for the determination of vitamin requirements in semisynthetic media. This medium had the following composition per 100 ml.: enzymatic casein hydrolyzate (5.0%), 10.0 ml.; sodium chloride, 100.0 mg.; potassium monohydrogen phosphate, 100.0 mg.; potassium dihydrogen phosphate, 100.0 mg.; sodium acetate, 50.0 mg.; ammonium chloride, 100.0 mg.; minerals X,* 0.25 ml. For use in preparing inocula, this medium was supplemented with 0.5% yeast extract. When used to determine vitamin requirements, a mixture of vitamins was added to give the following final concentrations in micrograms/100 ml. of medium: thiamine . HCl, pyridoxine . HCl, pyridoxal . HCl, calcium pantothenate, niacin, riboflavin, inositol, choline, and *p*-aminobenzoic acid, 50.0; pyridoxamine . HCl, 10.0; folic acid, 0.2; biotin, 1.0. The medium was dispensed in test tubes in 10.0 ml. amounts.

*$MgSO_4 \cdot 7H_2O$, 10.0 g.; $FeSO_4$, 0.273 g.; $MnSO_4 \cdot H_2O$, 0.379 g.; distilled water, 250 ml.

After autoclaving, 0.25 ml. of sterile dextrose solution (0.4 g./ml.) was added per tube. Significant growth failed to occur in this medium in the absence of yeast extract or vitamins.

In preliminary trials with synthetic media, results using the medium of Campbell and Williams (6), and in a medium containing 24 amino acids in proportion to their percentage occurrence in 0.5 g. of casein (10), were not consistent with all strains. Consequently, in order to confirm the vitamin requirements apparent in semisynthetic media, and to make observations on the amino acid requirements, a synthetic medium was devised (Table I).

TABLE I

SYNTHETIC MEDIUM EMPLOYED FOR DETERMINING THE VITAMIN AND AMINO ACID REQUIREMENTS OF *Bacillus coagulans*

Constituent	Amount	Constituent	Amount
L-Lysine	38.0 mg.	Ca pantothenate	50.0 μg.
DL-Isoleucine	32.0 mg.	Pyridoxine . HCl	50.0 μg.
L-Leucine	52.5 mg.	Pyridoxal . HCl	50.0 μg.
DL-Valine	35.0 mg.	PABA	50.0 μg.
L-Arginine	16.5 mg.	Pyridoxamine . HCl	10.0 μg.
DL-Threonine	20.0 mg.	Biotin	1.0 μg.
DL-Methionine	12.5 mg.	Folic acid	2.0 μg.
L-Cystine	1.5 mg.	Glucose	1.0 g.
DL-Phenylalanine	22.5 mg.	NH_4Cl	100.0 mg.
L-Histidine	12.0 mg.	Na acetate	50.0 mg.
L-Tryptophan	4.5 mg.	NaCl	100.0 mg.
L-Glutamic acid	102.0 mg.	K_2HPO_4	100.0 mg.
Thiamine . HCl	50.0 μg.	KH_2PO_4	100.0 mg.
Riboflavin	50.0 μg.	Minerals X	0.25 ml.
Niacin	50.0 μg.	Distilled water	100.0 ml.

This medium contained amino acids in the approximate concentrations that would be obtained using 0.5% enzymatic casein hydrolyzate based on information obtained from the manufacturer. The complete medium was dispensed in 5.0 ml. amounts in test tubes prior to autoclaving.

The vitamin requirements were established by deletion of vitamins from the complete medium. Pyridoxine, pyridoxal, and pyridoxamine were deleted as a group. Folic acid and *p*-aminobenzoic acid (PABA) were deleted both alone and together. The remaining vitamins were deleted individually.

Amino acid requirements were studied by deleting the acids from the synthetic medium (prepared without riboflavin) in groups according to their type, as follows:

Group 1.—DL-Valine, L-leucine, DL-isoleucine, and DL-threonine.

Group 2.—DL-Methionine and L-cystine.

Group 3.—L-Glutamic acid.

Group 4.—DL-Phenylalanine, L-tryptophan, and L-histidine.

Group 5.—L-Arginine and L-lysine.

Media were sterilized by autoclaving for 15 minutes under 15 lb. steam pressure. All tests were performed in duplicate on at least 2 different days,

the complete medium serving as control. None of the strains developed in synthetic media when the nitrogen equivalent as ammonium sulphate was substituted for amino acids. Trace growth, which often occurred in control tubes or deficient media in spite of repeated washing of inocula, failed to increase on prolonged incubation and gradually disappeared in subculture. This precluded the possibility of adaptation or mutation.

Results

Vitamin Requirements in a Semisynthetic Medium

All strains of *B. coagulans* tested required biotin and thiamine for significant growth (Table II). In addition, strain 1604 required niacin. Folic acid and PABA had little effect on growth when deleted individually, but when deleted together, growth of most strains was reduced. Therefore, it appeared that folic acid and PABA were interchangeable. Recently, this same relationship was reported for a single strain of *B. coagulans* (3).

TABLE II

GROWTH OF *Bacillus coagulans* UPON DELETION OF VITAMINS FROM SEMISYNTHETIC MEDIUM

Vitamins deleted	% transmittance	
	Typical of 20 cultures	Culture 1604
None	37.1	34.9
Thiamine	*96.5**	*98.6*
Pyridoxine group	38.9	41.3
Pantothenate	45.2	38.8
Niacin	41.9	*99.1*
Riboflavin	38.8	33.4
Biotin	*96.2*	*96.7*
Inositol	39.5	33.7
Choline	40.3	34.0
PABA	40.1	35.7
Folic Acid	39.3	31.0
PABA, folic acid	51.5	29.0
All	*96.6*	*99.2*

*All results *italicized* where vitamin deficiencies are evident.

Tests were conducted to determine the ability of each strain to grow in the presence of minimal vitamin supplements. The design of this experiment may be noted in Table III. While most strains grew in the presence of the minimal vitamin mixtures, growth in general was reduced.

Vitamin Requirements in a Synthetic Medium

The results obtained here (Table IV) were, in some respects, similar to those obtained in the semisynthetic medium in that all strains required biotin and thiamine, and strain 1604 required niacin. They differed greatly, however, in that 19 of 22 strains, including ATCC 12245, required folic acid or PABA. Since none of these strains required folic acid or PABA in the semisynthetic

TABLE III

GROWTH RESPONSE OF *Bacillus coagulans* TO MINIMAL VITAMIN MIXTURES IN SEMISYNTHETIC MEDIA

Vitamins present	% transmittance	
	Typical of 20 cultures	Culture 1604
All 10 (control)	42.3	45.7
Thiamine, niacin, pantothenate, biotin	57.1	42.5
Thiamine, niacin, biotin	62.0	47.4
Thiamine, biotin, pantothenate	60.9	*99.0**
None	*96.2*	*99.3*

*All results *italicized* where definite vitamin deficiencies are evident.

TABLE IV

GROWTH OF *Bacillus coagulans* UPON DELETION OF VITAMINS FROM SYNTHETIC MEDIUM

Vitamins deleted	Group*		
	I	II	III
	% transmittance		
None	51.7	53.6	45.5
Thiamine	*94.9*†	*98.8*	*95 7*
Riboflavin	51.9	52.8	45.8
Niacin	51.9	*96.9*	45.0
Pantothenate	52.3	55.1	44.6
Pyridoxine group	52.3	52.0	45.6
Biotin	*96.9*	*98.5*	*95.3*
Folic acid	51.9	55.9	45.4
PABA	53.6	55.4	47.2
PABA, folic acid	*94.8*	59.3	68.4
All	*95.9*	*98.2*	*96.0*

*Group I —Typical of 19 cultures.
Group II —Culture 1604.
Group III—Typical of two cultures.
†All results *italicized* where a vitamin requirement is evident.

medium, it was concluded that the enzymatic casein hydrolyzate either provided factors which partially replaced the folic acid or PABA requirement of *B. coagulans* or precursors from which this organism could synthesize these vitamins.

Amino Acid Requirements

The amino acid requirements were generally nonspecific at 37° C. Of 22 strains tested, only four required any specific group(s) (Table V). The deletion of Group 1 (DL-valine, L-leucine, DL-isoleucine, and DL-threonine) from the medium had a pronounced effect on the growth of only one strain (No. 132). Four strains (C2253, 88, 43P, and 132) required glutamic acid. The response of strain 43P was variable in the absence of the aromatic, heterocyclic, and the diamino monocarboxylic acids.

TABLE V

GROWTH OF *Bacillus coagulans* UPON DELETION OF AMINO ACIDS FROM SYNTHETIC MEDIUM

Amino acids deleted	Group*			
	I	II	III	IV
	% transmittance			
None	46.3	54.3	57.0	44.0
DL-Valine, DL-threonine, L-leucine, DL-isoleucine	55.0	57.7	*86.7*†	55.2
DL-Methionine, L-cystine	53.4	60.5	64.7	57.4
L-Glutamic acid	59.3	*96.2*	*88.4*	*98.0*
DL-Phenylalanine, L-tryptophan, L-histidine	47.4	57.6	72.6	Variable
L-Arginine, L-lysine	47.8	57.3	61.5	Variable
All	*95.1*	*95.0*	*96.7*	*98.6*

*Group I —Typical of 18 cultures.
Group II —Typical of two cultures.
Group III—Culture 132.
Group IV—Culture 43P.
†All results *italicized* where a definite amino acid deficiency is evident.

Stimulation

With one exception, better growth of the strains of *B. coagulans* studied was obtained in the semisynthetic medium containing the enzymatic hydrolyzate (Nutritional Biochemicals Corp.) than in the synthetic medium partially derived from it. This apparent stimulation was mainly attributable to buffering action resulting from the relatively high concentration of acetate incorporated in the enzymatic hydrolyzate during manufacture. Strain ATCC 7050, which failed to show this evidence of stimulation, was found to be sensitive to high concentrations of acetate.

Discussion

The study of the vitamin requirements of *Bacillus coagulans* in semisynthetic and synthetic media showed that two strains (711 and ATCC 7050) required only biotin and thiamine. These observations agree with those of Knight and Proom (11) and Proom and Knight (12), who noted that these were the only growth factors required by 16 strains (including ATCC 7050) of this organism in semisynthetic and synthetic media.

The results obtained in the synthetic medium support those of Campbell and Williams (6) in that many strains may require folic acid in addition to biotin and thiamine. Although three strains did not require folic acid, two of them (711 and ATCC 7050) were stimulated by this vitamin. This suggests that folic acid is of general importance in the nutrition of *B. coagulans*.

The present study has shown that the test medium used is an important factor in nutritional studies with *B. coagulans*. This is evidenced by the general requirement for folic acid or PABA in the synthetic medium but not

in the semisynthetic. The fact that a difference in this respect was not observed by Proom and Knight (12) may be a reflection either of the difference in their methods of isolation or of variation in the incidence of particular strains, depending upon geographical location. In this connection, it will be noted that all of our fresh isolates required folic acid while strain ATCC 7050, the reference strain employed by Knight and Proom (11) and Proom and Knight (12), did not require this vitamin.

Only one strain tested was shown to require niacin in the present study. Except for single strains reported to require niacin by Campbell and Williams (6) and by Cleverdon *et al.* (8), no further reports in this respect were found in the literature. Therefore, the requirement for this growth factor appears rare within the species.

The amino acid requirements of *B. coagulans* were found to be generally non-specific. However, this is not surprising. Campbell and Williams (6) found that specificity in the amino acid requirements shown by *B. coagulans* at higher temperatures of incubation (45° and 55° C.) may disappear at a lower incubation temperature (36° C.). Also, Bhat and Bilimoria (5) noted that certain metabolites necessary for growth at 55° C. were non-essential at 37° C. Nevertheless, the organisms still required organic nitrogen. Apparently this species possesses synthesizing mechanisms which function at low temperatures but not at high.

This study indicated that glutamic acid may play an important role in the nutrition of *B. coagulans*, either as an essential metabolite or as a growth supplement. Allen (1) and Andersen and Werkman (2) noted that glutamic acid may be important in the nutrition of this organism.

The inconsistent results obtained using casamino acids as a nitrogen source, and the failure to obtain good growth of all strains in the medium of Campbell and Williams (6), are not readily explainable. That our synthetic medium is considerably higher in total nitrogen than that of Campbell and Williams, and further differs in the type and proportion of amino acids present, might account for the different results. This idea is in line with the results of Proom and Knight (12), who obtained growth of 16 strains of *B. coagulans* in a 14-amino acid medium, but not in a medium containing only 7 amino acids.

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EXTRACELLULAR LYSINE PRODUCTION BY VARIOUS FUNGI[1]

M. RICHARDS[2] AND R. H. HASKINS

Abstract

Cultures of over 600 fungi were screened for the production of extracellular free lysine in submerged agitated culture on a medium containing urea, cerelose, and mineral salts. Sixty-three of the isolates produced free lysine in quantities sufficient to be detected by a turbidimetric bio-assay procedure, and 12 of these produced 5–15 γ lysine per milliliter of culture filtrate. With two strains of *Ustilago*, yields were increased to 200–300 γ lysine per milliliter culture mixture by adjustment of cultural conditions. The addition of ammonium acetate or of acetic acid, or the increasing of the amount of urea in the medium from 0.06% to 0.6%, resulted in increased yields from certain organisms.

Introduction

Certain of the amino acids are known to be in short supply in many staple foods, particularly those of cereal origin (6), and lysine is the primary limiting amino acid in wheat protein (7) when it is used as a food.

Supplementation of cereal foods with the necessary amino acids is not widely practiced as yet, partly because of the high cost of the amino acids. In consequence, plant protein does not yet adequately replace animal protein in the diet. Lysine, particularly, has been far too expensive for consideration in food fortification (8) although recent reports (1, 2) indicate that a two-stage fermentation process yields L-lysine at a price considerably lower than that of the chemically synthesized DL-lysine.

This development and the present work indicate the potentialities of both fungi and bacteria for the economic production of amino acids which have bearing on nutrition and the world's food supply. The product may be added to the diet in a pure form or as a dried whole fermentation culture mixture.

A program of screening various fungi was designed to detect those capable of accumulating free lysine in their culture filtrates. The best-yielding strains were further tested by modifications of the cultural conditions.

Methods

Test Organisms

Cultures tested for lysine production were transferred to Difco potato dextrose agar plates. Portions of the resulting colonies were blended with sterile distilled water in semimicro Waring blendors, and the resulting suspensions used to inoculate 100 ml. aliquots of an autoclaved nutrient

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[2]National Research Council of Canada Postdoctorate Fellow, 1954–55. Present address: Istituto Superiore di Sanita, Viale Regina Elena, 299, Rome, Italy.

solution contained in 500 ml. Erlenmeyer flasks. Unless otherwise stated, this nutrient solution consisted of a mineral base (K_2HPO_4 0.3 g., KH_2PO_4 0.2 g., $MgSO_4.7H_2O$ 0.2 g., NaCl 0.1 g., $CaCl_2.2H_2O$ 0.1 g., $FeSO_4.7H_2O$ 0.015 g., $ZnCl_2$ 0.00025 g., distilled water to 1 liter), a carbon source (glucose monohydrate 5%), and a nitrogen source (urea 0.06%).

The inoculated flasks were incubated at 28° C. on a rotary shaker operating at 225 r.p.m. with a radius of motion of 1 in. Unless otherwise stated, cultures were examined for lysine production after 5 days' growth.

Assay Organisms

The organisms used were *Leuconostoc mesenteroides* P60 (PRL 1410, ATCC No. 8042) or *Neurospora crassa* Beadle 4545A (PRL 1409, ATCC No. 10335). They were maintained on Bacto-Micro Assay culture agar by stab inoculation. The maintenance of the assay organisms and the subculturing for preparation of cell suspensions, etc., were carried out essentially as recommended in the Difco Manual (3) for the use of Bacto-Lysine Assay Medium.

Assay Procedure

The directions given in the Difco Manual (3) for assay of lysine were followed for most of the experiments. Except where otherwise indicated, 1 ml. of culture liquor was added to 10 ml. of lysine assay medium for the assay. After required incubation, 1 ml. was removed and diluted with distilled water to 10 ml. before turbidity was estimated on a Coleman nephelometer.

The assay methods for lysine using *N. crassa* and *L. mesenteroides* (3) were compared to determine which would be the more useful under our conditions. When using *N. crassa* as the assay organism a standard curve ranging from 20–100 γ/ml. was obtainable and 3–6 days' incubation was required. With *L. mesenteroides* as the assay organism, a standard curve ranging from 0.5–25 γ/ml. was obtainable and the assay was completed in less than 12 hours. *L. mesenteroides* was therefore used as the assay organism and its response to lysine was measured turbidimetrically since acidimetric determinations were found to vary considerably at the lower concentrations.

Lysine hydrochloride was used to prepare the standard solutions which were calculated as "free" lysine per unit volume.

Growth of the *Leuconostoc* assay organism was never observed in the lysine assay medium without the addition of lysine or of a culture liquor presumably containing lysine. Acetate or α-aminoadipic acid had no effect on the growth of the assay organism.

Results and Discussion

Survey

Isolates from about 600 fungi were grown and screened for the production of lysine which accumulated extracellularly in the culture liquor. Of these fungi, 25 were actinomycetes, 14 phycomycetes, five ascomycetes, 47 basidiomycetes, 414 Fungi Imperfecti, and 114 were unidentified isolates.

Small quantities of free lysine were found in the culture liquor of 63 of the organisms (two actinomycetes, four phycomycetes, 18 basidiomycetes, 31 Fungi Imperfecti, and eight unidentified). In 12 of these, the concentrations detected ranged from 5–15 γ lysine/ml. of crude culture liquor (Table I) and the organisms were investigated further. In the remainder of the 63, the yields did not exceed 2 γ/ml.

TABLE I

AMOUNTS OF FREE LYSINE ACCUMULATED IN CULTURE LIQUOR BY VARIOUS FUNGI GROWING IN AGITATED UNMODIFIED MEDIUM CONTAINING UREA, CERELOSE, AND MINERAL SALTS

PRL No.	Organism	Conc. of free lysine in culture liquor (γ/ml.)
79	*Gliocladium roseum*	10
184	Stilbellaceae (?)	15
377	*Streptomyces* sp.	25
527	*Darluca filum*	15
535	*Aspergillus ochraceous*	10
1092	*Ustilago maydis*	30
1137	*Trichoderma* sp.	10
1159	*Chaetomium* sp.	15
1229	*Ustilago maydis*	15
1259	*Monochaetia unicornis*	25
1269	*Monochaetia unicornis*	15
1414	*Penicillium* sp.	15

L. mesenteroides failed to grow on the lysine assay medium to which culture filtrates from certain of the cultures had been added, even when lysine was added and the pH was adjusted to a level suitable for *L. mesenteroides* growth. It is probable that the assay organism was inhibited by antibiotic substances produced by the fungi concerned.

Effect of Age of Inoculum

Lysine production by each of the 10 selected organisms was not markedly affected by the age (1–8 weeks) of the agar cultures from which the homogenized inocula were prepared.

Effect of Variations in the Medium

The 10 best lysine producers were grown on the specified medium but with varying amounts of the constituents. When the urea was increased from 0.06% to 4.0% the medium became so basic that the organisms did not grow at all, or only very poorly, and no detectable lysine was produced. Sterilizing the urea separately, preferably by filtration, and adding it aseptically to the sterilized and cooled medium provided media with pH values more suitable for growth of the test organisms and resulted in increased lysine yields. With one organism tested, *Ustilago maydis* PRL 1092, using 0.6% urea in the

culture mixture rather than 0.06% resulted in yields of 200–300 γ free lysine/ml. culture broth. Sampling was periodical and it was obvious that these high yields were held for several days and were not transient as were the low yields of other cultures.

Yields of lysine by some isolates (e.g. from PRL 1092 and 1229) were increased significantly by the addition of 0.1% ammonium acetate to the medium. Part of this increase was due to increase of ammonia nitrogen in the medium. The addition of 0.1% α-aminoadipic acid to the medium did not seem to have any appreciable effect on the lysine yield. The addition of acetic acid, however, resulted in yields of over 200 γ lysine/ml. broth. The effect on lysine production of the use of buffers to control the pH of the cultures was undetermined because the buffers interfered with the growth of the assay organism. The better lysine production obtained on addition of *N* acetic acid to the medium may have been directly due to the acetic acid itself or merely to the resulting more favorable pH of the medium.

With some cultures, using the medium containing urea, cerelose, and a mineral base under apparently identical conditions, the yields obtained varied from run to run. However, of the fungi tested, the *Ustilago maydis* cultures, PRL 1092 and 1229, were the most successful and consistent producers. The lysine yields of these strains were much increased by the addition of more urea to the culture medium but it was then necessary to sterilize the urea separately by filtration; an unfavorable basic reaction was not always avoided, even with this step, because of the liberation of an excess of ammonia from urea by the growing cultures. However, yields of 200–300 mg. lysine per liter culture filtrate were obtained in this way.

The best yielding strains were selected for further studies, the results of which have been published elsewhere by Dulaney *et al.* (5) for *Ustilago maydis* and Dulaney (4) for *Ustilago maydis* and *Gliocladium* sp.

Acknowledgments

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LECITHINASE PRODUCTION BY STRAINS OF BACILLUS CEREUS FR. AND FR. PATHOGENIC FOR THE LARCH SAWFLY, PRISTIPHORA ERICHSONII (HTG.)[1]

DONN J. KUSHNER[2] AND A. M. HEIMPEL[3]

Abstract

Strains of *Bacillus cereus* pathogenic for the larch sawfly, *Pristiphora erichsonii*, produce an enzyme that breaks down purified egg lecithin and the phospholipids of egg yolk and insect tissue. A study, using specific chemical techniques to measure lecithinase activity, of the relation between lecithinase production and pathogenicity supports an earlier postulate that lecithinase plays a role in the pathogenicity of *B. cereus* for the larch sawfly.

Introduction

Heimpel (6) has recently postulated that lecithinase plays a role in the invasion and destruction of the larch sawfly by certain strains of *Bacillus cereus* Fr. and Fr. This theory was based on three principal observations: first, *Bacillus* species and *B. cereus* strains that did not produce lecithinase were not pathogenic for the larch sawfly; second, there appeared to be a correlation between the relative amounts of lecithinase produced by strains of *B. cereus* grown on an artificial medium and the pathogenicity of these strains for the insect; and third, the histopathology of the *B. cereus*-infected insects indicated that lecithinase action, but not lipolytic or proteolytic action, had taken place.

In Heimpel's studies, lecithinase activity was measured indirectly, using the egg-yolk reaction (11). We have now reinvestigated the relationship between lecithinase production and pathogenicity, measuring lecithinase activity by a more direct, chemical method. This was necessary, since an investigation of the egg-yolk reaction itself has shown that it is not in all cases specific for measuring the lecithinase activity of *B. cereus* cultures (9). Specific methods, suitable for measuring the small amounts of lecithinase produced by our strains of *B. cereus*, have now been worked out. Results are presented here dealing with the action of *B. cereus* enzymes on purified lecithin and on the phospholipids in egg yolk and insect tissue; and with the relation between pathogenicity and lecithinase production.

Materials and Methods

The techniques used for growing bacteria, testing their pathogenicity, and preparing concentrated bacterial enzymes have been described (1, 6). Egg-

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yolk saline (0.2% NaCl) used as a substrate for phospholipase activity was prepared as before; egg lecithin was purified by the method of Hanahan, Turner, and Jayko (4).

Enzymic breakdown of lecithin and other phospholipids was measured by following the release of acid-soluble phosphorus. The incubation mixture used when purified lecithin served as the substrate contained 0.1 ml. 0.25 *M* borate buffer, pH 7.2; 0.05 ml. 0.1 *M* $CaCl_2$; *B. cereus* enzyme or culture medium from which the bacteria had been removed by centrifugation; water to 1.5 ml.; and 0.5 ml. 2.5% lecithin. The materials were mixed in a glass-stoppered tube; 0.2 ml. toluene and 0.2 ml. ether were added, and the tube was tightly stoppered, shaken, and incubated 2 hours at 27° C. The reaction was stopped by the addition of 2.0 ml. 20% trichloroacetic acid; the contents of the tube were then cooled in ice and filtered through Whatman No. 50 paper. The filtrate was extracted three times with 5 ml. portions of ether, and the residual ether was removed by heating for 5 minutes at 55°–60° C. Total phosphorus in the filtrate was determined, after digestion with H_2SO_4 and H_2O_2, by the Fiske–SubbaRow (3) method. Corrections were made for the phosphorus present in the enzyme preparations used. The amount of phosphorus released, up to 50 μg., proved to be a linear function of the enzyme concentration.

TABLE I

ACTION OF *B. cereus* LECITHINASE ON THE PHOSPHOLIPIDS OF EGG YOLK AND INSECT TISSUE

Material	Fraction	μg. phosphorus found	
		Control	After enzyme action
Egg-yolk saline	Acid-soluble	7.2	62.2
	Lipid	120.8	60.8
	Remainder (nucleic acid + protein)	2.4	2.4
Larch sawfly larval homogenate	Acid-soluble	76.4	88.4
	Lipid	14.4	3.0
	Remainder	14.2	14.2

Conditions: Egg-yolk saline: 0.5 ml. egg-yolk saline; 1.0 ml. 0.05 *M* borate buffer, pH 7.2; 1.0 ml. *B. cereus* enzyme; 0.55 ml. 2% NaCl; water to 6.0 ml. Incubated 4 hours at 37° C.

Larch sawfly homogenate: 60 mg. homogenate; 0.5 ml. borate buffer; 0.5 ml. *B. cereus* enzyme; water to 2.0 ml. Incubated 2.5 hours at 37° C.

Water replaced the *B. cereus* enzyme in the controls.

When egg-yolk saline or insect homogenates served as substrates of lecithinase activity, incubations were carried out as shown in Table I. Following incubation, the proteins and remaining phospholipids were precipitated by the addition of 2.0 ml. 20% trichloroacetic acid and removed by centrifuging. The increase in phosphorus in the supernatant was then determined as before.

Results and Discussion

Phospholipase Activity of B. cereus Enzymes

Neither the *B. cereus* culture supernatants nor any of the concentrated enzyme preparations attacked purified lecithin in aqueous medium, and it was necessary to add organic solvents to initiate lecithinase activity. Ether alone could activate lecithinase, the optimal amount of ether under our conditions being 0.2 ml. Slight changes in the concentration of ether, however, as might be caused by evaporation, produced a wide variation in activity. The mixture of ether and toluene finally used gave lower but more reproducible activity measurements.

It should also be noted that Kates (7) found that plant plastid phospholipases, which split choline from lecithin, can only do so in the presence of ether. Hanahan and Vercamer (5) observed that lecithinase from *Clostridium perfringens* Type A, the action of which is similar to that of *B. cereus* lecithinase, attacks lecithin dissolved in 98% ether – 2% ethyl alcohol.

The *B. cereus* enzyme released acid-soluble phosphorus both from egg-yolk saline and from insect tissue homogenates in an aqueous medium. Analysis of the various phosphorus-containing fractions (10), before and after enzyme action, showed that the phosphorus released came only from the lipid fraction (Table I). Besides hydrolyzing the phospholipids of the larch sawfly larvae, the *B. cereus* enzyme also acted on those of forest tent caterpillar larval midgut.

Phosphoryl choline (determined by phosphate and choline (8) analysis after enzymic hydrolysis (12)) was the major phosphorus-containing compound released from egg-yolk phospholipids. From the reported substrate specificity of the *B. cereus* lecithinase, it seems likely that cephalin as well as lecithin in egg yolk was attacked.

The Relation of Lecithinase Production to Pathogenicity

Enough insects were collected to permit adequate pathogenicity tests of three *B. cereus* strains. Two series of tests were carried out, 1 month apart. The results obtained are shown in Table II, with some of the earlier findings of Heimpel included for comparison. The three strains appear to have approximately the same absolute and relative pathogenicities as reported earlier. In the first of our tests, using third- and fourth-instar larvae, strain Mu-3055 showed less pathogenicity than in the second test, using late fourth- and fifth-instar larvae, but no generalizations can yet be drawn from this finding.

The relative lecithinase production by the same three strains, growing in nutrient broth, was also measured (Table III). Strain Mu-3055 produced about twice as much lecithinase as strain PR-1017, whereas strain PR-1011 produced hardly any. This pattern of lecithinase production differs somewhat from that reported earlier. The former measurements, by the egg-yolk reaction (6), indicated that the lecithinase production of strain PR-1017 was almost as great as that of Mu-3055, and this result was also obtained when the lecithinase activity of the cultures used here was measured by the egg-yolk

TABLE II

MORTALITY CAUSED BY THREE STRAINS OF *B. cereus* FED TO THE LARCH SAWFLY

Expt.	Strain	No. tests	No. larvae	Total No. dead	Mortality due to bacterium	
					No.	%
1*	PR-1011	2	62	11	5	8
	PR-1017	4	162	56	42	26
	Mu-3055	5	216	76	59	27
2†	PR-1011	6	295	48	37	12
	PR-1017	6	260	83	67	21
	Mu-3055	6	273	118	109	40
3‡	PR-1011	2	36	8	3	10
	PR-1017	5	108	39	38	36
	Mu-3055	3	56	22	21	38
4§	PR-1011	4	200	36	7	3.5
	PR-1017	4	200	102	62	31.0
	Mu-3055	4	200	91	81	40.5

*With third- and fourth-instar larvae.
†With fourth- and fifth-instar larvae.
‡From Heimpel (6) Table V; with fourth- and fifth-instar larvae.
§From Heimpel (6) Table VI; with fourth- and fifth-instar larvae.

TABLE III

AN EXPERIMENT ON LECITHINASE PRODUCTION IN NUTRIENT BROTH BY THREE *B. cereus* STRAINS

Strain	Tube	Lecithinase activity (μg. P)
PR-1011	1	5
	2	1
	3	3
	4	1
PR-1017	1	26
	2	25
	3	20
	4	14
Mu-3055	1	51
	2	55
	3	39
	4	46

NOTE: Bacteria of each strain were subcultured from agar plates into tubes containing 20 ml. sterile nutrient broth. After 20 hours' growth at 37° C., 1.0 ml. of each culture supernatant was assayed for lecithinase activity.

Bacteria in tubes 1 and 2 were those used in Expt. 1; in tubes 3 and 4 were those used in Expt. 2 (See Table II).

A similar pattern of activity was found when egg-yolk saline instead of lecithin was used as the substrate.

reaction. As observed before, strain PR-1011 had the lowest lecithinase production as well as the lowest pathogenicity.[4]

We believe that these results are consistent with the theory that lecithinase production is an important factor in *B. cereus* pathogenicity. Direct chemical

[4]Measurements of the lecithinase production of strain PR-1011 by the egg-yolk reaction were difficult to interpret. This strain now produces a heat-stable substance(s) that causes turbidity in egg-yolk saline without having lecithinase activity (9). If the egg-yolk reaction is carried out in double-strength broth, the turbidity production by the heat-stable substance is inhibited, but turbidity production by lecithinase still takes place.

tests have shown that pathogenic strains of *B. cereus* do produce lecithinase and that the lecithinase acts on the phospholipids of insect tissue. The two most pathogenic strains produce much more lecithinase than does the third, which has low pathogenicity. Of the two most pathogenic strains, Mu-3055, which produces more lecithinase, caused, at least in one test, the greater mortality. A linear relation between lecithinase production and pathogenicity is not necessarily to be expected.

Dolby and Macfarlane (2) have recently reported, and considered the implications of, the relation between lecithinase production and pathogenicity in different strains of *Clostridium perfringens* (Wilsdon). For our own results, a more extended discussion of this relation does not at present appear profitable. We have measured production of lecithinase by bacteria growing in nutrient broth. In the mid-gut of the infected larch sawfly, bacteria grow under (presumably) quite different conditions; a comparison between lecithinase production in one environment and pathogenicity in the other is, therefore, necessarily arbitrary. More information on the influence of the environment on lecithinase production appears necessary, and a study of this problem is now under way.

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PROTEINASE BIOSYNTHESIS BY STREPTOCOCCUS LIQUEFACIENS

II. PURINE, PYRIMIDINE, AND VITAMIN REQUIREMENTS[1, 2]

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Abstract

Proteinase biosynthesis by *Streptococcus liquefaciens*, strain 31, may be obtained from an entirely synthetic medium. Adenine and uracil seem to satisfy best the purine and pyrimidine requirement while pyridoxal and riboflavin is the combination of vitamins that leads to maximal enzyme biosynthesis. Biotin and folic acid together, or thiamine, have an inhibitory effect on enzyme synthesis. The amino acid requirements for optimum activity are unaffected by the reduced number of vitamins and nitrogen bases present in the final medium as compared to the initial medium. A reduction in the number of vitamins (to pyridoxal and riboflavin), however, showed that only adenine and uracil gave the best response to the purine and pyrimidine demands for biosynthesis; prior to this, in the presence of a complete vitamin pool, several combinations of nitrogen bases yielded equal enzymatic activity. When proteinase synthesis is plotted against time, enzyme formation ceases at about 3 hours. The shape of the curve indicates a straight-line relationship after an initial lag period of 90 minutes.

Introduction

An earlier report by Rabin and Zimmerman (5) described some of the nutritive and environmental factors necessary for proteinase biosynthesis by resting cells of *Streptococcus liquefaciens*, strain 31. The present paper deals with the results obtained from a continuation of that previous study. Specifically, vitamin, purine, and pyrimidine requirements have been defined. The interrelationships of these constituents to each other and to the required amino acids (5) also have been investigated.

Materials and Methods

Most of the materials and methods have already been detailed (5). In essence, cultures of *Streptococcus liquefaciens*, strain 31, were prepared for the biosynthetic studies by growing the cells in the casein semisynthetic medium (CSM) and transferring the washed population to the modified synthetic medium (MSM). After a suitable period of incubation, an estimate for newly-formed, extracellular proteinase was obtained by measuring the amounts of solubilized tyrosine produced on a casein substrate with an aliquot of the supernatant material of the incubation flasks.

Substitutions, as described in the next section, were made in the MSM in order to further define the nutritional requirements of proteinase formation.

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[2]Authorized for publication on November 26, 1956, as paper No. 2105 in the journal series of the Pennsylvania Agricultural Experiment Station.
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A new procedure, associated only with this report, was used in experiments dealing with rates of enzyme biosynthesis. Twenty milliliters of the final inoculum were added to 180 ml. of medium containing nutrients for 200 ml. Five milliliter aliquots were removed at each sampling time and, to stop the reaction, pipetted into tubes holding 0.05 ml. of 1.0 *M* sodium fluoride (5). These aliquots were then assayed.

Acid-cleaned glassware was used for all experiments concerned with determining vitamin requirements.

Experimental Results

Purine and Pyrimidine Requirements

The nitrogen bases normally present in the MSM (guanine, adenine, and uracil) were employed in various combinations to determine the purines and pyrimidines necessary for proteinase synthesis. The data in Table I indicate that adenine, guanine, and uracil, adenine and uracil, guanine and uracil, or guanine alone, are capable of maintaining a high level of biosynthesis. Adenine exerts a negative influence on guanine when both purines are used together (see Flask 3). Adenine and uracil, although no better than several other groupings tested, were arbitrarily chosen to represent the nitrogen bases in the MSM for future experiments.

The addition of xanthine and thymine (Experiment II, Table I) to the MSM had a slightly inhibitory effect.

Vitamin Requirements

Besides the normal complement of vitamins (pyridoxine, niacin, calcium pantothenate, riboflavin, folic acid, and biotin) in the MSM, pyridoxal, thiamine, and B_{12} were also tested for their ability to sustain enzyme synthesis.

TABLE I

EFFECT OF PURINES AND PYRIMIDINES ON PROTEINASE FORMATION

Flask	Nitrogen bases*	μg. tyrosine solubilized in 60 min.	% activity
	Experiment I		
1	Adenine, uracil, guanine	625	100
2	Adenine, uracil	665	106
3	Adenine, guanine	308	49
4	Guanine, uracil	700	112
5	Adenine	360	57
6	Guanine	650	104
7	Uracil	295	47
8	None	320	51
	Experiment II		
1	Adenine, uracil	480	100
2	Adenine, uracil, xanthine, thymine	420	88

*The concentration of the nitrogen bases is at a level of 0.25 mg. in each flask containing 50 ml. of medium.

TABLE II

THE VITAMIN REQUIREMENTS FOR PROTEINASE BIOSYNTHESIS

Vitamin*	Flasks																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Pyridoxine	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pyridoxal	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Riboflavin	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	−
Niacin	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	−
Ca pantothenate	+	+	+	+	+	−	+	+	+	+	+	+	−	−	−	−	−
Folic acid	+	+	+	+	+	+	−	+	+	+	−	−	−	−	−	−	−
Biotin	+	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−	−
Thiamine	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	−
B_{12}	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−
μg. tyrosine solubilized in 60 min.	645	645	0	615	625	675	655	665	835	680	722	709	730	697	790	534	50
% activity	100	100	0	95	97	105	102	103	130	105	112	110	113	108	123	83	8

*The vitamin concentrations employed are as follows: pyridoxine, pyridoxal, riboflavin, niacin, Ca pantothenate, thiamine, 1 μg. per ml.; B_{12}, 0.1 μg. per ml.; folic acid, 0.01 μg. per ml.; biotin, 0.001 μg. per ml.

A preliminary experiment had revealed that pyridoxamine gave levels of activity equivalent to those of pyridoxal. The data in Table II demonstrate that vitamins are essential for biosynthesis (see Flasks 1 and 17). That maximum enzyme production may be accomplished in the presence of only two vitamins, pyridoxal and riboflavin, is illustrated by a comparison of Flasks 15 and 16 to the remaining Flasks in Table II.

Several additional aspects of Table II are noteworthy. The removal of thiamine from the vitamin pool results in an increase of enzyme synthesis (Flasks 9, 15), thus demonstrating an inhibitory effect on the part of this substance. Omission of biotin and folic acid also results in synthesis stimulation (Flask 11) whereas the single removal of either of these vitamins has no effect on the system (Flasks 7, 8). Although pyridoxine is quite capable of contributing to growth and enzyme synthesis in the CSM, it apparently has none of this ability in the MSM.

The vitamin content of the MSM was now reconstituted so that for future experimental work it contained only pyridoxal and riboflavin.

Interrelationship of Purine, Pyrimidine, Vitamin, and Amino Acid Requirements

The experimental procedure had necessitated an altering of the ingredients of the MSM whenever the essential nutrilites in each group were determined.

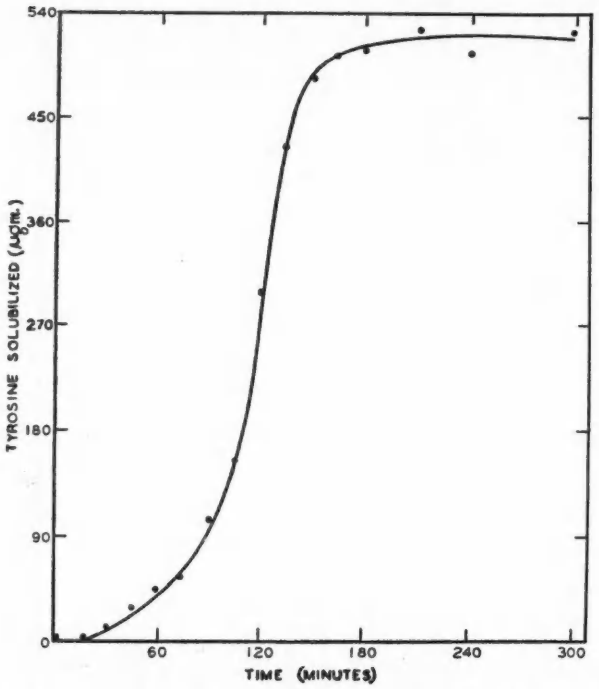

FIG. 1. The rate of proteinase biosynthesis in a completely synthetic medium.

In view of this fact, it seemed reasonable to assume that a change of content in any of the categories of nutrients tested might have led to a new arrangement of essential substances. The nitrogen bases were the first group of compounds rechecked in the MSM to test this hypothesis. Surprisingly, more definitive results were obtained; now adenine and uracil consistently promoted the greatest amount (by slightly over 10%) of proteinase biosynthesis. The percentage activity of all the other combinations tested were almost identical with those reported in Table I.

All 12 essential amino acids (5) were also retested. The results showed that these compounds were still necessary and to the same degree.

Rate of Proteinase Biosynthesis

Fig. 1 is a graphic representation of the rate of proteinase biosynthesis in the final MSM. An examination of this curve reveals that after the initial lag of 90 minutes a straight-line relationship results which is finally terminated by a cessation of enzyme formation after about 3 hours.

Discussion

Not only are the nitrogen bases required for growth by purineless and pyrimidineless mutants of *Escherichia coli*, but Pardee (3) has shown that their presence is also necessary for enzyme synthesis by these mutants. It is difficult to assess the role of these compounds in growth with respect to the nutrition of the lactic acid organisms. Niven and Sherman (2) in discussing this point, for instance, stated that all their cultures grew in the absence of purines and pyrimidines. When these substances were included in the medium, however, the rate of growth was greatly enhanced in most cases.

Snell (7) also reported, for *Streptococcus faecalis*, that purine bases were not required in a medium if adequate amounts of folic acid were present. Later, working with the same organism, but a different medium, Rabinowitz and Snell (6) noted that growth of the organism in the presence of adenine, guanine, and uracil was occasionally inhibited, but never stimulated. In a recent article, Maciasr (1) states that purine bases will inhibit growth in a phosphate buffer.

It is obvious from the data in Table I that, even if purines and pyrimidines are not required for growth, some such substances are necessary for optimum proteinase biosynthesis. Since no comparative rate studies were made, there is no evidence as to whether maximum enzyme synthesis could have occurred without these compounds, if the time of incubation had been prolonged. Nevertheless, it seems logical to assume that nucleic acid synthesis must occur in order to get enzyme formation.

The fact is of interest that, of all the vitamins tested, only pyridoxal and riboflavin were required. The enterococci are known generally to require more vitamins than these two for growth (2). It is possible that our organism is capable of synthesizing some of the vitamins found to be non-essential, or that our washed cells retained a sufficient vitamin supply to carry on enzyme

biosynthesis. However, for pyridoxal and riboflavin, it is more attractive to assume that both are more directly concerned in enzyme synthesis. Certainly pyridoxal, as a coenzyme to the transaminating enzymes, might play an important role in such synthesis. If the necessity of riboflavin could be solely attributed to its role in hydrogen transfer, then one would expect niacin to be required also.

The greater stimulation by pyridoxal rather than pyridoxine is, of course, not surprising (8). The growth of *Streptococcus liquefaciens* in the CSM (casein semisynthetic medium) can be adequately explained in terms of the pyridoxine being converted to pyridoxal and pyridoxamine through autoclaving. What is anomalous, however, is the fact that proteinase biosynthesis was achieved in experiments prior to the vitamin studies in the MSM (modified synthetic medium) containing pyridoxine instead of pyridoxal. The observation during these early experiments that enzyme synthesis increased up to a point and then decreased as the vitamin stock solution aged suggests the conversion of pyridoxine to the more active form in the stock solution. In the vitamin studies themselves, where no enzyme was synthesized in the presence of pyridoxine, the vitamins were freshly prepared in separate solutions so as to minimize or avoid any opportunity for interconversion. As a result of the vitamin studies, freshly prepared vitamins were employed for all succeeding experiments.

The curve representing the time-course of enzyme formation shows that most of the proteinase is synthesized at a linear rate. The initial non-linear portion of the curve may result from any one of a number of environmental or intracellular conditions (4).

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POLYHYDRIC ALCOHOL PRODUCTION BY OSMOPHILIC YEASTS: EFFECT OF OXYGEN TENSION AND INORGANIC PHOSPHATE CONCENTRATION[1]

J. F. T. Spencer and Ping Shu

Abstract

A study was made of the effects of oxygen tension and inorganic phosphate concentration on the production of glycerol, arabitol, and ethanol by a strain of osmophilic yeast. Increasing the oxygen tension increased the yields of glycerol and arabitol and decreased the yield of ethanol, while increasing the concentration of inorganic phosphate had the opposite effect.

The changes in yields of products with increasing phosphate concentration were most pronounced at a partial pressure of oxygen of 280 mm. of mercury. At lower oxygen tensions the yields of polyols were low and the yield of ethanol was high at all concentrations of phosphate, while at very high oxygen tensions the opposite effect was observed. When the ethanol yield was high, the respiratory quotient was also high. These results are discussed in relation to the oxidative phosphate-lack theory of the Pasteur effect.

Introduction

One of the characteristics of the osmophilic yeasts is the large increase in glycerol yield associated with increased aeration of the culture medium (11). This provides a practical means of varying the glycerol yield over a wide range. This phenomenon also provides another approach to the study of the Pasteur effect. With *Saccharomyces cerevisiae* this effect manifests itself in a decreased rate of glucose utilization and in reduced ethanol yield as the rate of aeration is increased. In experiments with osmophilic yeasts a similar change in ethanol yield and glucose utilization rate is observed as the aeration rate is increased, and is accompanied by the increase in glycerol yield previously mentioned.

The decrease in rate of glucose dissimilation and ethanol yield with increased aeration, as observed in *S. cerevisiae* fermentations, has been explained by Johnson (3) and Lynen (5) as resulting from the reduction of the intracellular concentration of inorganic phosphate. According to this theory, glucose is utilized more efficiently in the presence of oxygen, more orthophosphate is esterified per mole of glucose dissimilated, and the concentration of inorganic phosphate is reduced. This inhibits glyceraldehyde-3-phosphate dehydrogenase, which requires inorganic phosphate, and reduces the rate of dissimilation of glucose.

Supporting evidence for this theory has been sought by studying the organic phosphates of yeast grown aerobically and anaerobically, as was done by Holzer and Holzer (2), or by investigating the activity of the citric acid

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cycle in yeast grown under these conditions. Aubert and Milhaud (1), using the latter method, found that the activity of the citric acid cycle was more intense in yeast grown aerobically, and stated that as a result, more phosphate esters should be formed and the concentration of orthophosphate reduced.

The opposite approach, by studying the effect of changing the extracellular orthophosphate concentration, has not been used in the study of the mechanism of the Pasteur effect, although the oxidative phosphate-lack theory predicts that changes in orthophosphate concentration should produce corresponding changes in the yields of fermentative products. The osmophilic yeasts are especially suited for such a study, since the scheme postulated for polyol formation (10) suggests that changes in the activity of glyceraldehyde-3-phosphate dehydrogenase, induced by changes in phosphate concentration, should alter the yields of all the major products of this fermentation. Since the Pasteur effect is of such importance in the production of glycerol by these organisms, the effects of changes in oxygen tension and orthophosphate concentration on their metabolic products were studied.

Materials and Methods

Strain P_3a of *Saccharomyces rouxii* (classified according to Lodder and Kreger-van Rij (4)), which was isolated in this laboratory (11), was used throughout these experiments. The culture was carried on slants of a medium containing 60% honey, 0.25% yeast extract, and 2–2.5% agar. Inoculum was prepared by incubating the culture 48 hours at 30° C. on a rotary shaker, in a medium containing 20% glucose, 1.0% yeast extract, and 0.2% urea, and 5% by volume was added. The basal medium for the experimental work contained 20% glucose, 1.0% yeast extract, and 0.23% urea, except in the experiments in the macrorespirometer, where the glucose level was 10.0% and the urea, 0.20%. To these media additions of disodium phosphate were made as shown in the different experiments. The yeast extract used contained some phosphorus compounds which amounted to 0.003 M. total phosphorus of which phosphate phosphorus was 0.0017 M. in the medium.

Fermentation Conditions

Preliminary studies of the effect of inorganic phosphate on polyol and ethanol production were made in 500-ml. Erlenmeyer flasks containing 50 ml. of medium incubated on a rotary shaker having an eccentricity of 1 in. at 230 r.p.m., at a temperature of 30° C. For the experiments in which the effects of oxygen tension on the fermentation were studied, the macrorespirometer of Shu (8) was used. In this apparatus any desired gas mixture may be used as atmosphere in the fermentation flasks, and a continuous record of the oxygen uptake during the fermentation is obtained. Six culture flasks can be used at one time on this equipment.

Analytical Methods

Inorganic phosphorus was determined colorimetrically with ammonium molybdate according to the method of Fiske and Subbarow as described by Umbreit *et al.* (12).

Total phosphorus was determined by oxidizing the sample with 10*N* sulphuric acid and then with hydrogen peroxide (Umbreit *et al.* (12)) and then proceeding as for inorganic phosphorus.

Total polyhydric alcohols were determined by oxidation with periodic acid and colorimetric determination of the formaldehyde formed with chromotropic acid reagent.

Glycerol and arabitol were separated by partition chromatography on water–celite columns and were then determined by the same method as for total polyols.

Reducing sugars were determined colorimetrically with the alkaline copper reagent of Somogyi (9) and the arsenomolybdate reagent of Nelson (7).

Ethanol was determined after distillation in a Conway microdiffusion cell by oxidation with acid potassium dichromate and determination of the excess dichromate with potassium iodide and sodium thiosulphate.

All the above methods, except those described by Umbreit *et al.* (12) are those compiled and described in detail by Neish (6).

Results

Ethanol yields decreased with increasing oxygen tension (Fig. 1) and increased with increasing phosphate concentrations (Table I and Figs. 2–5). The increase in ethanol yield with increased phosphate concentration was

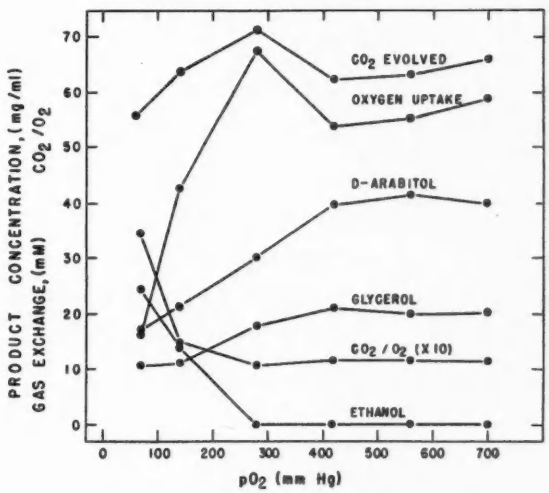
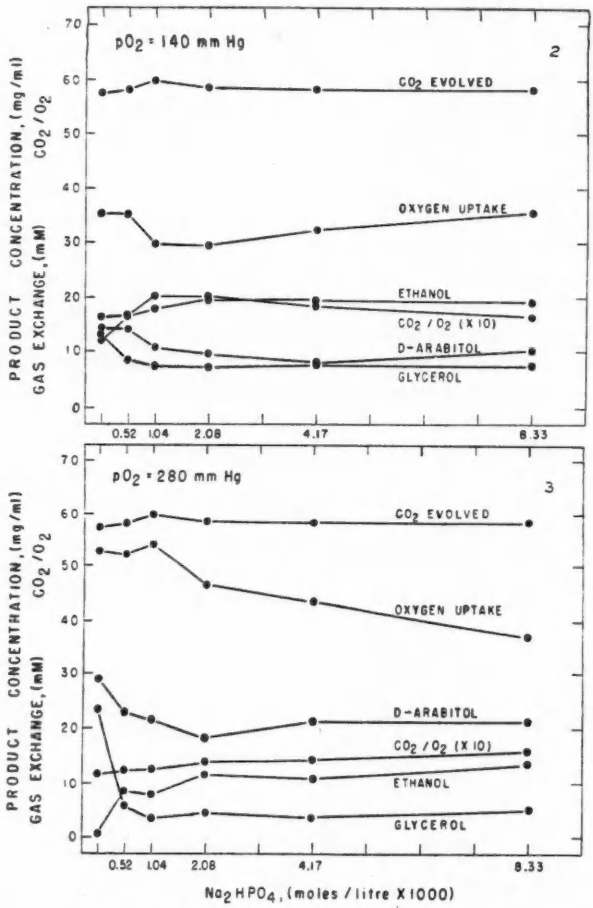
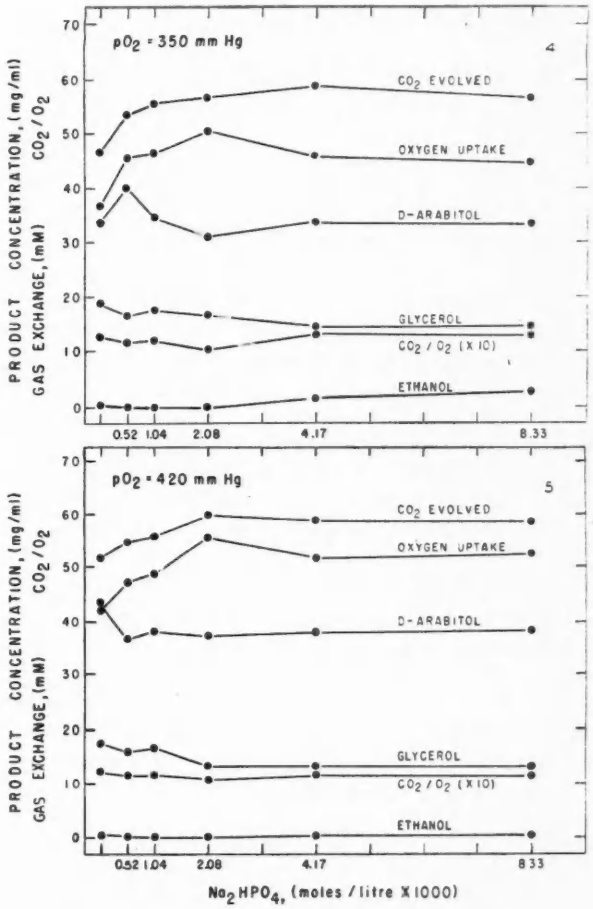


FIG. 1. Effect of oxygen tension in the absence of added phosphate.



FIGS. 2 and 3. Effect of added phosphate. FIG. 2: pO_2 = 140 mm. mercury; FIG. 3: pO_2 = 280 mm. mercury.



FIGS. 4 and 5. Effect of added phosphate. FIG. 4: pO_2 = 350 mm. mercury; FIG. 5: pO_2 = 420 mm. mercury.

TABLE I

EFFECT OF DISODIUM PHOSPHATE ON POLYHYDRIC ALCOHOL PRODUCTION

Basal medium 20% glucose + 1.0% yeast extract. Incubated on a rotary shaker of 1 in. eccentricity, speed 230 r.p.m., temperature 30° C.

Na_2HPO_4, moles/liter (× 1000)	Maximum rate of glucose utilization, mg./ml./day	Polyhydric alcohols, mg./ml.		Ethanol, mg./ml.	
		2 days	4 days	2 days	4 days
Nil	67	30	82	17	6
0.52	74	28	67	22	9
1.04	86	26	50	26	14
2.08	99	25	31	35	21

most noticeable at pO_2 of 280 mm. of mercury (Fig. 3). At lower oxygen tensions the ethanol yield was so high at the lowest phosphate concentration that the relative increase in yield was low. At higher oxygen tensions the ethanol yield remained very small, though a slight increase in yield could still be seen at a pO_2 of 350 mm. mercury (Fig. 4).

Polyhydric alcohol yields increased with increasing oxygen tension (Fig. 1) and decreased with increasing phosphate concentration (Figs. 2–5). The increase in arabitol yield (Fig. 1) with increased oxygen tensions had not been observed in the earlier studies (11). As with ethanol the greatest change in glycerol yield as affected by phosphate concentration was found at a pO_2 of 280 mm. mercury (Fig. 3).

Oxygen uptake and *carbon dioxide evolution* reached a maximum at a pO_2 of 280 mm. mercury, then decreased as the oxygen tension was increased further (Fig. 1). The changes in gas exchange with increasing phosphate concentration depended on the oxygen tension in the gas phase. At a pO_2 of 140 mm. of mercury (Fig. 2) the oxygen uptake and carbon dioxide evolution remained approximately constant at all phosphate concentrations used and at a pO_2 of 280 mm. of mercury (Fig. 3) the oxygen uptake decreased with increasing phosphate concentration. At higher oxygen tensions (Figs. 4 and 5) the oxygen uptake and carbon dioxide evolution increased with increasing phosphate concentration, the rate of increase being greatest at lower concentrations of phosphate.

The *respiratory quotient* increased with increasing phosphate concentrations at low oxygen tension but at higher ones remained nearly constant at a value slightly greater than one (Figs. 2–5). There was a positive correlation between ethanol yield and respiratory quotient, the latter increasing markedly at the higher ethanol yields (Fig. 6).

The rate of glucose utilization increased with increasing phosphate concentration (Table I).

Cell weights increased only slightly with increasing phosphate concentration at oxygen tensions at or below 280 mm. of mercury. At higher oxygen

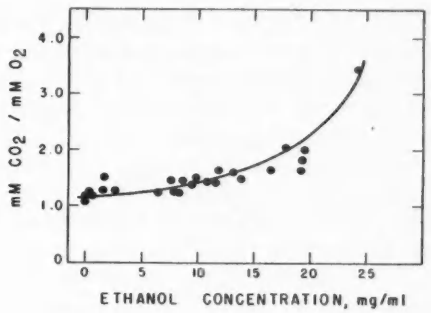


FIG. 6. Relation between respiratory quotient and ethanol yield.

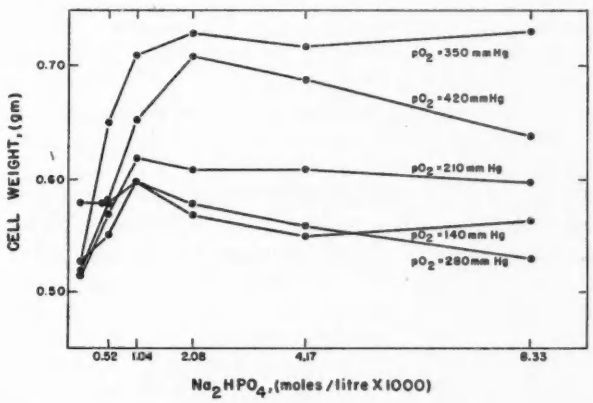


FIG. 7. Effects of phosphate concentration and oxygen tension on cell yields.

tensions there were large increases in cell yields as the concentration of added phosphate increased from 0 to 0.002 *M* after which the changes were less marked (Fig. 7).

Discussion

These experiments show in more detail than previous work (11) the effect of oxygen tension on the production of polyhydric alcohols, and show in addition the relationship between inorganic phosphate concentration and oxygen tension in the metabolism of osmophilic yeasts.

In the absence of added phosphate the yields of polyols increased with increasing oxygen tension. The oxygen tension at which maximum yields were reached, however, was considerably higher than that at which the peak in oxygen uptake and carbon dioxide evolution occurred. The ethanol yield reached zero at the latter oxygen tension also, and the respiratory quotient declined to its lowest value, so that the change-over from anaerobic to aerobic glucose dissimilation was probably complete at that point. The positive

correlation between ethanol yield and respiratory quotient illustrates this phenomenon further as the respiratory quotient is at its lowest when the ethanol yield is zero, and increases with increased ethanol production. No reason can be given, however, for the decrease in oxygen uptake and carbon dioxide evolution at higher oxygen tensions.

In the presence of added orthophosphate the effects expected were observed. With increasing orthophosphate concentration the ethanol yield increased and the yield of polyhydric alcohols decreased. This effect was most noticeable at a pO_2 of 280 mm. of mercury, which represented a critical oxygen tension. At pO_2's greater or less than this, the changes in yields of products with changes in phosphate concentration were much smaller. There was also an increased respiratory quotient and a higher rate of glucose utilization as the orthophosphate concentration was increased.

These observations show that the inorganic phosphate concentration in the medium affects the balance between the pathways of glucose dissimilation in this organism.

The effects observed can be explained by assuming that orthophosphate concentration is the limiting factor in the activity of glyceraldehyde-3-phosphate dehydrogenase as follows:

(*a*) Increased enzyme activity results in conversion of more triose phosphate to ethanol, with an accompanying reduction in glycerol formation.

(*b*) Reduction in the concentration of glyceraldehyde-3-phosphate due to increased enzyme activity as in (*a*) results in lowered arabitol yields, since the postulated mechanism of arabitol formation (10) requires glyceraldehyde-3-phosphate as an acceptor for active glycolaldehyde from fructose-6-phosphate.

(*c*) Increased ethanol concentration is associated with higher values of the respiratory quotient, since 1 mole of carbon dioxide is formed anaerobically per mole of ethanol.

(*d*) Conversion of more triose phosphate to ethanol, as described in (*a*), increases the rate of glucose utilization.

This explanation is in accordance with the oxidative phosphate-lack theory proposed by Johnson (3) and Lynen (5), and extends this theory. The increased cell yield observed at the higher concentration of phosphate and high oxygen tensions is also explained by this theory. Under these conditions neither oxygen nor phosphate is limiting; oxidative processes, with their higher efficiency in high energy phosphate synthesis, may be expected to predominate; and the organic phosphates thus produced are available to contribute energy for the synthesis of cell material.

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INHIBITION OF NONFOLIC-ACID-REQUIRING ORGANISMS BY FOLIC ACID ANTAGONISTS: MECHANISM OF REVERSAL[1]

VIRGINIA MIMS MCGLOHON, BARBARA HOEVET PETERSON, AND O. D. BIRD

Abstract

Although folic acid antagonists have been reported to be unable to inhibit nonfolic-acid-requiring organisms, it was found that several such antagonists were quite active inhibitors of *Lactobacillus arabinosus* and several species of yeasts when grown in minimal media. The growth inhibition produced under these circumstances was not reversed by compounds related to folic acid but was reversed by thiamine and vitamin B_6 compounds. A rapidly developing physiological adaptation caused by the presence of thiamine and vitamin B_6 apparently was the mechanism by which these organisms overcame the inhibitory effects of folic acid antagonists. 6-Mercaptopurine antagonized this reversal and thus was strongly synergistic with folic acid analogues under certain conditions.

Introduction

It was reported by Woolley and Pringle (11) that microorganisms requiring folic acid as a growth factor are inhibited by folic acid analogues, while those not requiring this vitamin are uninhibited even by rather large amounts of these analogues. Franklin *et al.* (7) succeeded in inhibiting the growth of *Escherichia coli* with very large amounts of 4-aminopteroylglutamic acid and reversing this inhibition with liver extract or thymidine. Recently Nickerson and Webb (9) observed that the inhibitory action of folic acid analogues on nonfolic-acid-requiring microorganisms was less in complex organic media than in simple chemically defined media. The studies reported here describe conditions under which nonfolic-acid-requiring microorganisms can be inhibited by rather small concentrations of folic acid analogues and demonstrate that this inhibition is not generally reversed by compounds related to folic acid, as in the case of folic-acid-requiring microorganisms, but by a combination of thiamine and pyridoxine acting by an entirely different mechanism.

Materials and Methods

The following organisms were used: *Saccharomyces cerevisiae* 69902, *Saccharomyces carlsbergensis* ATCC 9080, *Kloeckera brevis* X-915, *Streptococcus faecalis* ATCC 8043, and *Lactobacillus arabinosus* ATCC 8014. Test cultures were grown in 16 mm. tubes, the total volume of each culture being 2.5 ml. The bacterial cultures were incubated in a stationary position at 37° C. for *S. faecalis* and 30° C. for *L. arabinosus*, while the yeast cultures were incubated at 30° C. on a shaker. Turbidity readings were made in a Lumetron colorimeter Model 400.

[1]Manuscript received January 16, 1957.
Contribution from Research Laboratories, Parke, Davis & Company, Detroit, Michigan, U.S.A.

Minimal media were developed for each organism studied. These media contained only those constituents, other than carbon and nitrogen sources and minerals, needed for growth, in concentrations just sufficient to give full growth. In the descriptions of these minimal media that follow, all indicated amounts of materials are amounts required per 100 ml. of double-strength medium.

The minimal medium for *S. cerevisiae* and *S. carlsbergensis* was that of Atkin *et al.* (1) with the vitamin mixture modified, 10 mg. Tween 80 added, and the pH adjusted to 6.0. For *S. cerevisiae* the vitamins added were 10 μg. pantothenic acid, 0.5 μg. biotin, and 120 μg. inositol. *S. carlsbergensis* medium contained 12 μg. pantothenic acid, 24 μg. pyridoxine, and 120 μg. inositol. *K. brevis* medium was that of Burkholder *et al.* (4) modified by omitting casamino acids, substituting glutamic acid for asparagine, and adding 10 mg. Darco G-60-treated peptone. Thiamine and pyridoxine were reduced to 1 μg. of each. The medium and the tubes containing the antagonists were sterilized separately by steaming 10 minutes. Thiamine was added aseptically after it was sterilized. For inoculum each yeast was grown in its respective medium indicated above, washed once with saline, and 0.4 mg. cells added to 100 ml. of double-strength medium when *S. cerevisiae* was used, or 2 mg. of cells was added when *K. brevis* or *S. carlsbergensis* was used, before the inoculum was dispensed into tubes.

Medium for *L. arabinosus* was as follows: Difco casamino acids (treated with 10% Darco G-60 at pH 3.0) 1 g.; dextrose 1.5 g.; sodium acetate 1 g.; *dl*-tryptophan 0.6 mg.; cysteine 20 mg.; asparagine 50 mg.; KH_2PO_4 100 mg.; K_2HPO_4 100 mg.; $MgSO_4.2H_2O$ 4 mg.; NaCl 2 mg.; pantothenic acid 5 μg.; nicotinic acid 16 μg.; biotin 50 μg.; *p*-aminobenzoic acid 200 mμg.; pH 6.8. Tubes containing medium and water were autoclaved 10 minutes at 15 p.s.i. to sterilize. Thiamine and antagonists were sterilized by making the first dilution with 70% ethanol and subsequent dilutions with sterile water, except 6-mercaptopurine, which was autoclaved with the medium. The inoculum was prepared by transferring *L. arabinosus* once in the above medium, then washing with saline, and diluting to a transmission reading of 65 on the Lumetron. One drop of this diluted inoculum was added to each tube. Tubes were incubated at 30° C. and turbidity readings made on the Lumetron colorimeter. Stock cultures were carried on Difco micro-assay culture agar.

S. faecalis medium was the same as that for *L. arabinosus* except for the following additions or changes: pantothenic acid 4 μg.; nicotinic acid 40 μg.; biotin 20 mμg.; folic acid 80 mμg.; no *p*-aminobenzoic acid.

The form of vitamin B_6 used in reversal studies with yeasts was pyridoxine. In studies involving *S. faecalis* and *L. arabinosus*, pyridoxal was used. This is because these respective forms of vitamin B_6 have been reported to be most active for the organisms indicated.

The inhibitory compounds used in this study, the sources from which they were received, if they were not made in our laboratory or purchased, and the abbreviations used are shown below:

6-Mercaptopurine (6-MP)

4-Aminopteroylglutamic acid (4-APGA)

2,4-Diamino-7,8-dihydropteridine (pteridine), Dr. E. C. Taylor, Princeton University

4,6-Diamino-1-(3,4-dichlorophenyl)-1,2-dihydro-2-methyl-S-triazine monohydrochloride (triazine), Dr. E. J. Modest, Children's Cancer Research Foundation, Boston, Mass.

Experimental Results

S. cerevisiae grown in minimal medium was found to be inhibited by relatively small amounts of all three types of folic acid antagonists described above. Whereas compounds related to folic acid will reverse the inhibitory effect of folic acid analogues in organisms requiring this vitamin, no reversal of the inhibitory effect of these analogues on *S. cerevisiae* was obtained with a large number of known compounds including the following, which were added in the indicated amounts per milliliter of medium: 100 μg. pteroylglutamic acid, 80 μg. leucovorin, 20 μg. *p*-aminobenzoic acid, 10 μg. *p*-aminobenzoylglutamic acid, 10 μg. pteroic acid, 10 μg. xanthopterin, 10 μg. diopterin. Combinations of adenine, guanine, and thymine or thymidine also gave no reversal. However, a small amount of yeast extract caused reversal, and strangely enough, a mixture of B vitamins also reversed the inhibition completely. This effect was narrowed down to two vitamins in the mixture—thiamine and pyridoxine (B_1 and B_6). Table I shows data indicating the effect of the three folic acid antagonists on *S. faecalis*, which requires folic acid in its growth medium, and on *L. arabinosus* and *S. cerevisiae*, which do not. Small amounts of folic acid in the form of pteroylglutamic acid (PGA) or leucovorin were able to reverse the effects of the inhibitors on *S. faecalis* but not on *L. arabinosus* or *S. cerevisiae*. On the other hand, B_1 plus B_6 reversed the effects of the inhibitors on the latter two organisms but had no reversing effect in *S. faecalis*.

The data indicating reversal of the inhibitory effects of three folic acid antagonists on *S. cerevisiae* by B_1 and B_6 are shown in Table II. B_1 alone had some effect on the growth of *S. cerevisiae* after longer incubation in the presence of inhibitory amounts of 4-APGA while B_6 alone had almost no effect. The reversal of the triazine required both B_1 and B_6. Since this organism produces B_1 and B_6 it is likely that these results obtained with 4-APGA only after long incubation are due to synthesis of one or both of these vitamins by the slowly growing yeast cells. Over either a long or short incubation period the addition of a combination of B_1 and B_6 reversed the inhibitory effects of all three of these antagonists in *S. cerevisiae*.

S. carlsbergensis has a B_6 requirement. With this vitamin supplied in the medium this yeast was inhibited by 4-APGA or the pteridine. This inhibition

TABLE I

INHIBITION OF FOLIC-ACID-REQUIRING AND NONFOLIC-ACID-REQUIRING ORGANISMS BY FOLIC ACID ANALOGUES

Analogue	*S. faecalis*	*L. arabinosus*	*S. cerevisiae*
	μg. analogue per ml. causing half inhibition		
4-APGA	0.0015	0.005	2.9
Pteridine	2.0	> 30	4.0
Triazine	0.002	0.10	3.0
	μg. pteroylglutamic acid per ml. causing reversal		
4-APGA	0.002 (C)	>100	>100
Pteridine	0.003 (C)	—	>100
Triazine	0.002 (C)	>100	>100
	μg. leucovorin per ml. causing reversal*		
4-APGA	0.002 (C)	>100	>100
Pteridine	0.0005 (EP)	—	>100
Triazine	0.0005 (EP)	5	>100
	Reversal by B_1 plus B_6 (μg. per ml.)		
		B_1 + B_6	B_1 + B_6
4-APGA	No reversal	0.10 + 0.01	0.04 + 0.01
Pteridine	No reversal	— —	0.10 + 0.10
Triazine	No reversal	0.02 + 0.01	0.10 + 0.10

NOTE: C = competitive inhibition.
EP = end product inhibition.
* = corrected for inactive form present.

was reversed by B_1. *K. brevis* requires both B_1 and B_6, and with sufficient of these vitamins for maximum growth, this organism was inhibited by the pteridine. After 40 hours' incubation *S. cerevisae* and *S. carlsbergensis* grew out in the presence of folic acid antagonists while *K. brevis* remained inhibited by the pteridine for more than 40 hours. Thus the organisms that synthesize their own B_1 or B_1 and B_6 were able to overcome the inhibition after a time, whereas the organism that could not synthesize these vitamins remained inhibited.

L. arabinosus grown in minimal medium, containing no B_1 or B_6, was found to be inhibited by much smaller amounts of 4-APGA than other workers have reported using richer media. Bardos *et al.* (2) reported 0.80 μg. per 5 ml. tube as the lowest inhibitory amount. Table III shows that as little as 0.01 μg. 4-APGA per millliliter gave complete inhibition, yet the inhibition caused by 150 times this amount was reversed by 1 μg. each of B_1 and B_6. There was no reversal by any form of folic acid. The triazine was also inhibitory, although at a higher concentration. Inhibition by the triazine was reversed to some extent by B_1 alone but B_1 and B_6 together gave more reversal. There was some reversal by large amounts of leucovorin, but none by PGA. Foley and Haley (6) found other triazines reversed by leucovorin using *L. arabinosus*.

TABLE II

REVERSAL OF FOLIC ACID ANTAGONISTS IN *S. cerevisiae* BY B_1 AND B_6

	Turbidity readings (% transmission)								
	Hours incubation								
	16	24	40	16	24	40	16	24	40
4-APGA (μg./ml.)		0			4			30	
Additions to medium*									
None	50	20	20	100	85	20	100	100	42
B_1	50	21	20	60	32	20	80	40	20
B_6	50	20	20	96	85	20	100	100	20
$B_1 + B_6$	40	20	20	40	20	18	50	20	18
Pteridine (μg./ml.)					4			30	
Additions to medium*									
None				85	55	20	100	100	25
B_1				85	35	20	100	80	20
B_6				88	60	20	100	95	20
$B_1 + B_6$				40	18	20	38	22	20
Triazine (μg./ml.)					10			40	
Additions to medium*									
None				100	95	20	100	95	20
B_1				100	65	20	100	90	20
B_6				100	100	20	100	100	20
$B_1 + B_6$				40	35	20	40	35	20

*1/μg. of each indicated compound per milliliter of medium.

TABLE III

REVERSAL OF FOLIC ACID ANTAGONISTS IN *L. arabinosus* BY B_1 AND B_6

	Turbidity readings (% transmission)									
	Hours incubation									
	24	24	40	72	24	40	72	24	40	72
4-APGA (μg./ml.)	0		0.01			0.03			1.5	
Additions to medium*										
None	25	90	80	55	100	100	70	100	100	70
B_1	25	70	30	20	100	20	20	100	100	20
B_6	25	90	75	50	100	100	80	100	100	100
$B_1 + B_6$	25	25	25	20	80	20	20	100	35	20
Triazine (μg./ml.)						0.2			0.6	
Additions to medium*										
None					85	85	20	100	100	50
B_1					40	20	20	60	20	20
B_6					85	85	20	100	100	50
$B_1 + B_6$					30	20	20	30	20	20

*1/μg. of each indicated compound per milliliter of medium.

TABLE IV

EFFECT OF 4-APGA ON PABA PRODUCTION IN *S. cerevisiae*

Expt. No.	Additions to medium per ml.	Yeast cells per ml., mg.	PABA formed	
			Per ml. culture, μg.	Per mg. cells, μg.
1	None	5.7	0.30	0.05
2	2 μg. 4-APGA	0.8	0.31	0.38
3	4 μg. 4-APGA	0.3	0.60	2.10
4	1 μg. B_1	6.0	0.31	0.05
5	1 μg. B_1 + 10 μg. 4-APGA	6.5	2.46	0.38
6	1 μg. B_1 + 20 μg. 4-APGA	5.7	14.07	2.47

It seemed possible that B_1 or B_1 plus B_6 might accomplish the reversal of folic acid analogues by stimulating de novo synthesis of folic acid which might be effective against these inhibitors in nonfolic-acid-requiring organisms whereas exogenous folic acid is not. To test this, the experiment outlined in Table IV was set up in which *S. cerevisiae* was grown alone and in the presence of two different amounts of 4-APGA. The weight of cell produced was determined and the amount of PABA produced by the cells was assayed using an *E. coli* mutant strain ATCC 9723*a* and the method of Lampen *et al.* (8). The presence of 4-APGA greatly reduced the yield of cells but PABA accumulated, presumably because it was not being fabricated into PGA. When the inhibitory effect of the 4-APGA was reversed by B_1 the growth inhibition was corrected and the yield of cells was normal but the accumulation of PABA continued, indicating that *S. cerevisiae* was not circumventing the block produced by 4-APGA by the production of excess folic acid. This was further checked by assaying microbiologically, by the method of Bird *et al.* (3), for total folic acid compounds present in *S. cerevisiae* cells grown in the presence and absence of B_1 plus B_6. No increase in folic acid content was found. This experiment was repeated using *L. arabinosus*. Here, also, there was no increase in folic acid caused by the addition of B_1 and B_6. However, the addition of PABA, which was not produced by *L. arabinosus* under the conditions of the experiment, increased the amount of folic acid formed in direct proportion to the amount of PABA added. This confirms the work of Sarett (10). But even this formation of endogenous folic acid had no effect on the reversal of 4-APGA.

Evidence that in nonfolic-acid-requiring organisms B_1 plus B_6 reverse the action of folic acid antagonists by influencing an alternate route of purine synthesis was obtained by experiments with 6-mercaptopurine (6-MP). Data illustrating such an experiment are presented in Table V. These data also demonstrate a remarkable synergism of 6-MP and 4-APGA in *S. cerevisiae* and *L. arabinosus* when cultured under the conditions used in these experiments. This synergism is evident in the minimal medium cultures of *S. cerevisiae* and *L. arabinosus* where the addition of 6-MP and 4-APGA together causes an increased efficiency of the antimetabolites. But it is much

TABLE V

SYNERGISM OF 6-MP AND 4-APGA: EFFECT OF PURINES AND VITAMIN B_1 PLUS VITAMIN B_6

Organism and medium	μg. cpd. per ml. causing half inhibition			
	Each present alone		Both present together	
	6-MP	4-APGA	6-MP	4-APGA
S. cerevisiae (18-hour incubation)				
Minimal medium	>500	4	80	1
Minimal medium plus B_1 and B_6	>500	20	80	1
L. arabinosus (40-hour incubation)				
Minimal medium	>50	0.008	2	0.005
Minimal medium plus B_1 and B_6	>50	>1.5	2	0.005
Minimal medium plus hypoxanthine	>50	0.020	>50	0.020
Minimal medium plus hypoxanthine plus B_1 and B_6	>50	>1.5	>50	>1.5

TABLE VI

EFFECT OF INCUBATION TIME ON SYNERGISM OF 6-MP AND 4-APGA

L. arabinosus in:	μg. per ml. causing half inhibition			
	Incubation time			
	18 hours		40 hours	
	Analogues present			
	6-MP	4-APGA	6-MP	4-APGA
Minimal medium				
Analogues alone	0.5	0.003	>50	0.02
Analogues together	0.2	0.002	0.8	0.004
Minimal medium plus B_1 and B_6				
Analogues alone	0.5	0.008	>50	>1.5
Analogues together	0.2	0.002	0.8	0.004

more noticeable in a medium containing B_1 and B_6, in which 4-APGA is not very effective because its inhibitory effect on nonfolic-acid-requiring organisms is reversed by these vitamins. But the presence of 6-MP eliminates this reversal of 4-APGA by B_1 and B_6 and still allows the same degree of synergism which existed in the absence of B_1 and B_6. The addition of hypoxanthine to minimal medium in which *L. arabinosus* is inhibited by 4-APGA alone causes a slight reversal of this antagonist. There is no synergism when both antagonists are added together to the minimal medium containing hypoxanthine. This is apparently because this purine reverses the effect of 6-MP and renders it unable to act synergistically with 4-APGA. Other purines have been found to act essentially the same in this respect. When both hypoxanthine and B_1 plus B_6 are added to the minimal medium there is very little inhibitory activity of the combined antagonists against *L. arabinosus*.

The influence of B_1 and B_6 on the synergism of 6-MP and folic acid antagonists is particularly evident when short and long incubation periods are compared. Such data are shown in Table VI where results for 18- and 40-hour incubations of *L. arabinosus* are given. 6-MP used alone is an active inhibitor of this organism over a short growth period but very large amounts of the compound are required to inhibit growth over a longer time. However, when both 6-MP and 4-APGA are used together inhibition of growth is maintained over a long incubation period with relatively small doses of both compounds, even when the medium contains B_1 and B_6.

Reversal of 4-APGA action against folic-acid-requiring organisms by folic acid is different in nature than the reversal of 4-APGA inhibition of nonfolic-acid-requiring organisms by B_1 and B_6. This is illustrated in Figs. 1 and 2. As shown in the former, an *S. faecalis* culture with added 4-APGA, but containing enough leucovorin to counteract its effect, grew as fast as one containing no inhibitor. This was not the case when the inhibitory effect of

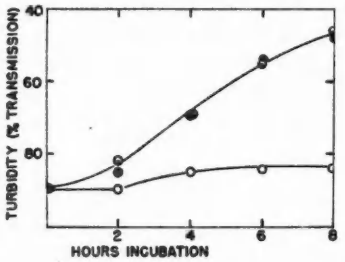

FIG. 1. Comparative growth response of *S. faecalis* growing in the presence of 10 mμg. leucovorin per ml. (●); 10 mμg. leucovorin + 4 mμg. 4-APGA per ml. (◒); and 1 mμg. leucovorin + 4 mμg. 4-APGA per ml. (○).

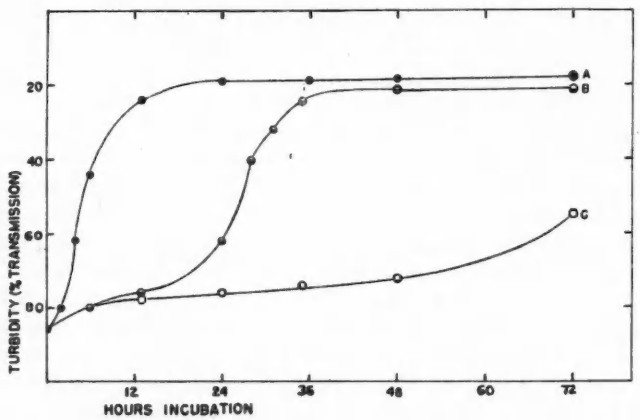

FIG. 2. Comparative response of *L. arabinosus* to B_1 and B_6 alone (A); to 4-APGA (C); and to 4-APGA + B_1 and B_6 (B).

TABLE VII

RECOVERY OF SENSITIVITY TO 4-APGA BY *L. arabinosus* CULTURE AFTER DEVELOPMENT OF RESISTANCE IN THE PRESENCE OF B_1 AND B_6

Description of *L. arabinosus* inoculum	4-APGA (μg. per ml.)					
	0	0.01	0.02	0.1	0.5	1.0
	Turbidity (% transmission)					
1. From culture containing 4-APGA plus B_1 and B_6	30	30	30	40	62	65
2. Same as (1) after one transfer in minimal medium	35	35	45	65	100	100
3. Same as (1) after three transfers in minimal medium	35	60	75	90	95	100
4. Control culture (B_1 and B_6 present. Never in contact with 4-APGA)	35	55	70	90	100	100

4-APGA on *L. arabinosus* was reversed by the presence of B_1 and B_6, as shown in Fig. 2. Here the culture containing added B_1 and B_6 did not grow out for a few hours after the control containing no inhibitor, but still grew much more quickly than the culture containing inhibitor with no added B_1 and B_6. It would appear that the effect of the B_1 and B_6 was to cause rapid development of resistance to the folic acid antagonist. Evidence in Table VII indicates that the development of resistance is a physiological adaptation and not a permanent mutation. After the third transfer of *L. arabinosus* in which this adaptation had been induced, through medium no longer containing 4-APGA or B_1 and B_6, the culture had regained its original sensitivity to this inhibitor.

Discussion

The experimental data presented seem to fit the scheme outlined in Fig. 3. According to this it is visualized that 4-APGA in nonfolic-acid-requiring organisms acts primarily to prevent the conversion of PABA into folic acid cofactors (Co-F), thereby causing an accumulation of PABA. These folic acid cofactors normally combine with purine precursors to form polynucleotides. It is at this point, where folic acid antagonists also exert an inhibitory

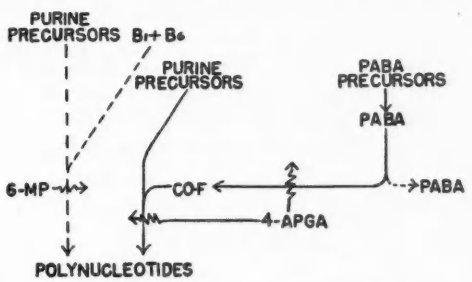

FIG. 3. Scheme showing possible relationship of the inhibitory actions of 4-aminopteroylglutamic acid and 6-mercaptopurine.

action as indicated in Table V, that hypoxanthine was shown to give some reversal of the inhibitory effect of 4-APGA. Since thiamine and pyridoxine or pyridoxal enable the growing organism to get around the blocks imposed by 4-APGA and other folic acid antagonists, this must be accomplished by the synthesis of polynucleotides from their precursors by a route (indicated in the figure by broken lines) which has a limited dependence on folic acid compounds but is mediated in some way by B_1 and B_6. This is evidently a rapid adaptive change, as indicated in Fig. 2. 6-MP apparently interferes with this alternate route since it is strongly synergistic with 4-APGA when B_1 and B_6 are present in the medium, a condition in which 4-APGA alone is quite inactive.

Elion, Singer, and Hitchings (5) found that a 6-MP-resistant strain of *L. casei* had an increased requirement for folic acid over that of the wild strain but required a reduced amount of a folic acid antagonist to inhibit it. This mutant had an altered purine metabolism and the authors concluded that it was able to utilize more than one pathway in the conversion of purines, one of which required less folic acid than the other. The alternate pathway requiring less folic acid could be the one described in this paper, which is dependent on B_1 and B_6 and which is blocked quite specifically, in the organisms studied, by 6-MP. This would account for the high degree of synergism between 6-MP and 4-APGA, since these two compounds apparently block alternate pathways in polynucleotide synthesis.

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A DESCRIPTION OF HAEMOPHILUS VAGINALIS AND ITS L FORMS[1]

C. R. Amies and S. A. Jones

Abstract

A new species of *Haemophilus*, *Haemophilus vaginalis*, has recently been incriminated as the cause of human non-specific vaginitis and urethritis. A very similar if not identical organism obtained from the same sources is the subject of this report. The name *H. vaginalis* is accepted for it. A systematic description of this organism is given, including microscopic and colonial appearances, nutritional requirements, biochemical properties, and pathogenicity to experimental animals. Immunological studies are not yet complete. Transformation of *H. vaginalis* into the L form of colony is described and illustrated in a series of photomicrographs. It is suggested that the pleuropneumonia-like organisms frequently encountered in genitourinary infections are, in fact, L forms of *H. vaginalis*. Attention must in future be concentrated on the normal bacterial phase and not on the L phase which is probably not pathogenic.

Introduction

The bacteriological examination of patients suffering from acute or subacute vaginitis has long been an unsatisfactory subject both for the laboratory worker and his clinical colleagues. Even when infections with *N. gonorrhoeae* and *Trichomonas vaginalis* have with reasonable certainty been excluded there still remains a large group of so-called non-specific infections, susceptible to some of the antibiotic drugs but liable to relapse, a source of much concern to the patient and sometimes a threat to marital happiness. A recent report by Gardner and Dukes (9) has thrown considerable light on this subject. From women suffering from this condition the authors have succeeded in isolating, in nearly every case examined, an organism to which they have given the name *Haemophilus vaginalis*. The appearance of this organism both in vaginal smears and in pure cultures is described and some of its biochemical properties are given. Of particular interest are their findings with 13 healthy volunteers inoculated with pure cultures of this organism: 10 remained completely normal, two developed symptomless infections lasting for 2 to 3 months, and one showed a mild vaginitis from which the organism was recovered in pure culture. A particularly valuable feature of this report is the detailed description of the clinical condition produced by this organism. As might be expected, the infection is transmitted to the male. In 45 of 47 cases investigated, the husbands of women suffering from this condition were found to harbor this same organism; and evidence is brought forward to support the belief that it may become more or less permanently established in the male urethra. The concept that emerges from the whole work is that *H. vaginalis* is a frequent inhabitant of the female genital tract; that it often assumes pathogenic characters; and that it is responsible for much of what has previously passed as "non-specific urethritis" in both sexes.

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Contribution from the Department of Bacteriology and the Provincial Laboratory of Public Health, University of Alberta, Edmonton, Canada.

In this laboratory, studies have been in progress for some time of an organism which we are now prepared to accept as *H. vaginalis* of Gardner and Dukes. The description here given is based upon 25 strains studied by conventional bacteriological and immunological methods. The transformation of these bacteria to the L forms through the agency of penicillin has also been studied. This last observation has led to certain conclusions which may be of far-reaching importance.

Materials and Methods

This Laboratory provides a diagnostic bacteriological service for medical officers of health, medical practitioners, and small hospitals throughout the northern half of the Province of Alberta. Patients thought to be suffering from gonorrhea are examined by the clinician and material for direct smears and culture is collected. All the clinical material examined in this investigation came to us in this way. It is unfortunate that no clinical records were available. Because of this we can only state that all the women from whom specimens were obtained had a cervical infection associated with a Grade III bacterial flora (1). Most of the specimens were received under a code number from the Social Hygiene Clinic or from a prison where persistent female offenders are detained for short sentences. Swabs were sent to the laboratory in the "transport medium" devised by Stuart, Toshach, and Patsula (20). This is an excellent method of maintaining the viability of delicate bacteria while the specimen is in transit. Most of the specimens were cultured on the same day that they were collected from the patient. In every case the bacterial flora was abnormal and inflammatory cells, mainly polymorphs, were present. The examination included cultivation on Peizer medium for isolation of *N. gonorrhoeae* (17, 22) and microscopic study of wet films to detect the presence of *Trichomonas vaginalis*.

For this special investigation the swabs were inoculated on a serum–yeast nutrient agar, the preparation of which is described below:

Basal medium

Peptone, Bacto tryptose	10 g.
Sodium chloride	5 g.
Agar	15 g.
Infusion from beef heart	1000 ml.
Phenol red	0.025 g.

For the sake of convenience, Bacto Blood Agar Base (Difco), which contains these nutrients in the required proportions is recommended. The pH is adjusted to 7.4 after the indicator is added.

The medium is lightly autoclaved and allowed to cool to about 45°. Sterile serum, human or sheep, is added to a concentration of 10%, and sterile yeast extract to 5%. The latter is prepared by the method of Cohen and Wheeler (2) except that fresh brewers' yeast is used instead of commercial dried yeast.

The use of fresh yeast greatly improves the growth-promoting properties of the extract. The medium is poured into Petri dishes, allowing 25 ml. per dish in order to provide a thick layer of agar. A similar medium without agar was used for fluid cultures; and the addition of 0.1% thioglycollic acid and 0.1% of agar to this fluid medium provided suitable conditions for anaerobic and microaerophilic growth. Culture plates were incubated at 37° in a "candle jar". In order to avoid overgrowth by other bacteria a strip of sterile filter paper (80 mm. × 10 mm.) soaked in penicillin solution, 10 units per ml., was placed on the surface of the medium immediately after inoculation. The penicillin diffused slowly through the medium and a concentration gradient was thus obtained. Once the organisms which are the subject of this report had been isolated in pure culture they were maintained without difficulty on serum–yeast medium or on freshly prepared chocolate agar.

For studying the L forms of the bacteria the methods described by Klieneberger-Nobel (11) and Dienes (3) were used. The phase microscope was invaluable for examining living unstained specimens.

Preliminary Studies

Our interest in the bacteriology of vaginitis arose from the fact that routine methods of examination often failed to demonstrate any known pathogen in spite of the presence, in direct smear preparations, of a markedly mucopurulent exudate and a heavy mixed bacterial infection. Like Gardner and Dukes we were impressed by the number of small Gram-negative bacteria in these smears. A special study was accordingly made of cervical exudate smears prepared at the Social Hygiene Clinic or the prison and forwarded to the laboratory for routine examination. The organism most frequently seen, often in very considerable numbers, was a small Gram-negative coccobacillus having a size range of 0.4 μ to 0.7 μ by 1.0 μ to 2.0 μ, frequently in pairs end-to-end or in short chains, sometimes in clumps or clusters consisting of many very small elements just on the limit of resolution by the microscope. Not infrequently the coccobacilli were seen to be attached to the surface of large flat epithelial cells. These are the bacteria described and illustrated in the report of Gardner and Dukes. A second morphological type of organism, less often seen, was a very slender straight or slightly curved rod with a size range of about 0.3 μ in width by 2 to 4 μ in length. These small rods occurred usually as single cells, not in clusters. Sometimes they were found within polymorphs and other phagocytic cells. These bacteria were indistinguishable from Koch-Weeks bacilli (*Haemophilus aegyptius*) as they appear in a smear of conjunctival exudate. These two morphological types, both of which proved to be *H. vaginalis*, are illustrated in Figs. 1 and 2.

Included in this preliminary study of stained smears were some obtained from cases of male urethritis. One thousand specimens in all were examined. Small Gram-negative coccobacilli were present in 41% of 829 smears of cervical exudate; and in 8% of 171 male non-gonococcal urethritis smears.

Isolation of *Haemophilus vaginalis*

As already stated, the specimens were obtained from women who had some degree of cervical infection associated with a Grade III bacterial flora. The presence of a heavy mixed growth on the culture plates was, therefore, only to be expected. The organisms most frequently found were coliform bacteria, staphylococci, fecal streptococci, and diplococci belonging to the tribe Mimeae (de Bord). Another group of organisms frequently present and very difficult to classify were pleomorphic Gram-variable bacteria appearing as cocci or small oval cells in clusters or short chains. These developed best under microaerophilic conditions. Some produced an area of α-haemolysis and greening on blood agar while others were not haemolytic. If further examination had been carried out some of these would probably have been classified as *Bacteroides*. All these organisms were considered to fall outside the sphere of the investigation and were accordingly studied no further.

The typical appearance of *H. vaginalis* colonies on a primary culture plate of serum yeast agar is illustrated in Fig. 3. The colonies measure 0.5 to 1.0 mm. in diameter, are circular, convex, transparent, and shiny, and are readily differentiated from staphylococci and coliforms growing on the same plate. They closely resemble colonies of fecal streptococci and confirmation by examination of stained smears is essential. Usually they do not grow in the immediate vicinity of the strip of filter paper impregnated with penicillin, but are readily detected in the area about 1.0 cm. from it where inhibition of most other bacteria is still evident.

From 371 cervical swab specimens inoculated on serum yeast agar plates containing a penicillin strip we obtained 19 strains of an organism which undoubtedly belonged to the genus *Haemophilus*. When the work of Gardner and Dukes became known to us we realized that we had isolated *H. vaginalis*. In this series, therefore, the organism was isolated in 5.1% of the specimens examined. There were also a few cases in which organisms of similar appearance were recognized in stained smears but subculture was not achieved. In addition, there were available five strains of a *Haemophilus* species isolated during the preliminary studies with Peizer medium. One strain obtained from a case of non-specific urethritis in a male patient is also included. The following description of *H. vaginalis* is based on the study of these 25 strains.

Description of *Haemophilus vaginalis*

On serum yeast agar plates inoculated with cervical exudate, *H. vaginalis* colonies appear after 24 hours' incubation as transparent convex shiny colonies ("dew drops") with a diameter of 0.5 to 1.0 mm. At 48 hours the center is often slightly raised, giving an umbonate appearance. The growth is still watery and difficult to collect on a platinum loop: when piled up with the loop the massed growth is translucent, colorless, and sometimes mucoid. The colonies do not show iridescence. On further incubation, some strains show fine refractile granules in the center of the colony, an appearance similar

to that seen in colonies of *H. influenzae* (14). This colonial morphology has been maintained unchanged for many months by transplanting the colonies every 3 or 4 days on the serum medium or on chocolate agar. The photographs reproduced in Figs. 4 and 5 illustrate these appearances.

The normal smooth *H. vaginalis* colony cannot with certainty be distinguished on morphological grounds from colonies of streptococci, a very important point for the bacteriologist contemplating routine diagnosis. Such streptococci frequently occur in large numbers on the primary culture plates in areas not reached by the penicillin.

When examined in stained smears, recently isolated strains are found to consist of small, thin, straight, or slightly curved rods resembling Koch-Weeks bacilli (*H. aegyptius*). The size is about 0.3 $\mu \times 2$ to 3 μ. They are invariably Gram-negative. After repeated transplants the bacteria become shorter and thicker or highly pleomorphic. Many strains eventually transform into very small coccobacilli. Some typical appearances are shown in Figs. 6 and 7. When the environment is unfavorable long filamentous forms appear and the colonies become rough (Figs. 8 and 9).

Growth in liquid media is poor in comparison with that obtained on a suitable solid medium. In serum yeast broth, an initial slight turbidity at 24 hours is later replaced by a deposit which is difficult to resuspend.

Growth Requirements

Luxuriant growth was obtained on the medium containing 10% of unheated serum and V factor in the form of yeast extract. The addition of 5% of defibrinated sheep blood to this medium definitely inhibited growth. On the other hand, chocolate agar prepared with sheep blood supported good growth. The serum yeast media were not improved by adding haematin in the form of Fildes' blood digest. This may perhaps be taken as evidence that *X* factor is not necessary for the growth of *H. vaginalis*, though it is possible that small amounts of *X* may have been present in the basal medium. Growth in synthetic media was not attempted. Bordet-Gengou medium, Loeffler's inspissated serum, Dorset egg, and ordinary blood agar failed to support growth.

Oxidation–Reduction Potential

The behavior of all 25 strains was the same in this respect. On the preferred medium, luxuriant growth was obtained by cultivation at 37° in a candle jar. This provided a humid atmosphere and some increase in CO_2 content. None of the strains would grow under completely anaerobic conditions. A condition of reduced oxygen tension was secured by evacuating the culture jar to 76 mm. Hg pressure and replacing the air by an inert gas. This gave no better results than cultivation in the candle jar. Moreover, in tubes of serum yeast broth containing thioglycollic acid and 0.1% agar, maximum growth occurred at or near the surface: no growth took place near the bottom of the tube.

Capsule Formation

The microscopic appearance of the organisms, both in smears of cervical exudate and in recently isolated pure cultures, sometimes suggested that a

thin capsule might be present. The watery or slightly mucoid consistency of the growth was in keeping with this observation. Attempts to demonstrate the presence of a capsule by the usual methods were, however, unsuccessful. No sign of a capsule-swelling reaction was seen when the organisms were brought into contact with sera containing the homologous antibody. The spontaneous agglutination of the organisms, to be described later, is further evidence against capsule production; but more information is needed before a definite opinion on this matter can be given.

Biochemical Reactions

Carbohydrate fermentation (using serum yeast agar containing 1.0% of the test substance) and other biochemical tests were carried out on 23 strains. All failed to ferment lactose, dulcitol, mannitol, trehalose, and rhamnose. Dextrose was attacked by all but one strain. Irregular results were obtained with the other carbohydrates. Of 23 strains tested the number found positive was as follows: maltose, 10; arabinose, 20; levulose, 11; xylose, 13; glycerol, 12; raffinose, 7; salicin, 14; sucrose, 10.

Ability to reduce nitrates to nitrites was demonstrated in every case. A positive indol reaction was given by two strains only. The oxidase reaction was invariably negative. Human, sheep, and rabbit erythrocytes were used to test for haemolysin production: none was demonstrated.

Sensitivity to Antibiotics

Soon after its isolation, each strain was tested for sensitivity to the usual antibiotics. The disk method was used. The results may be summarized as follows:

Penicillin:	sensitive to 1.0 unit	13 strains
	insensitive to 100 units	10 strains
Streptomycin:	sensitive to 4 mcg.	22 strains
	insensitive to 4 mcg.	1 strain
Tetracycline group:	sensitive to 10 mcg.	23 strains
	insensitive to 10 mcg.	0 strains
Chloromycetin:	sensitive to 8.0 mcg.	23 strains
	insensitive to 8.0 mcg.	0 strains

FIG. 1. Film of exudate from a case of acute "non-specific" vaginitis. Gram stain. Small diplobacillary forms of *H. vaginalis* undergoing phagocytosis by polymorph cells.

FIG. 2. Film of exudate from another case of acute "non-specific" vaginitis. *H. vaginalis* is here shown as a small slender slightly curved rod, not easily stained.

FIG. 3. Part of the surface of a serum yeast agar culture plate showing "dew-drop" colonies of *H. vaginalis* together with staphylococci and coliforms. The edge of the penicillin strip is seen on the left.

FIG. 4. Colonies of *H. vaginalis* on serum yeast agar: 48 hours at 37°.

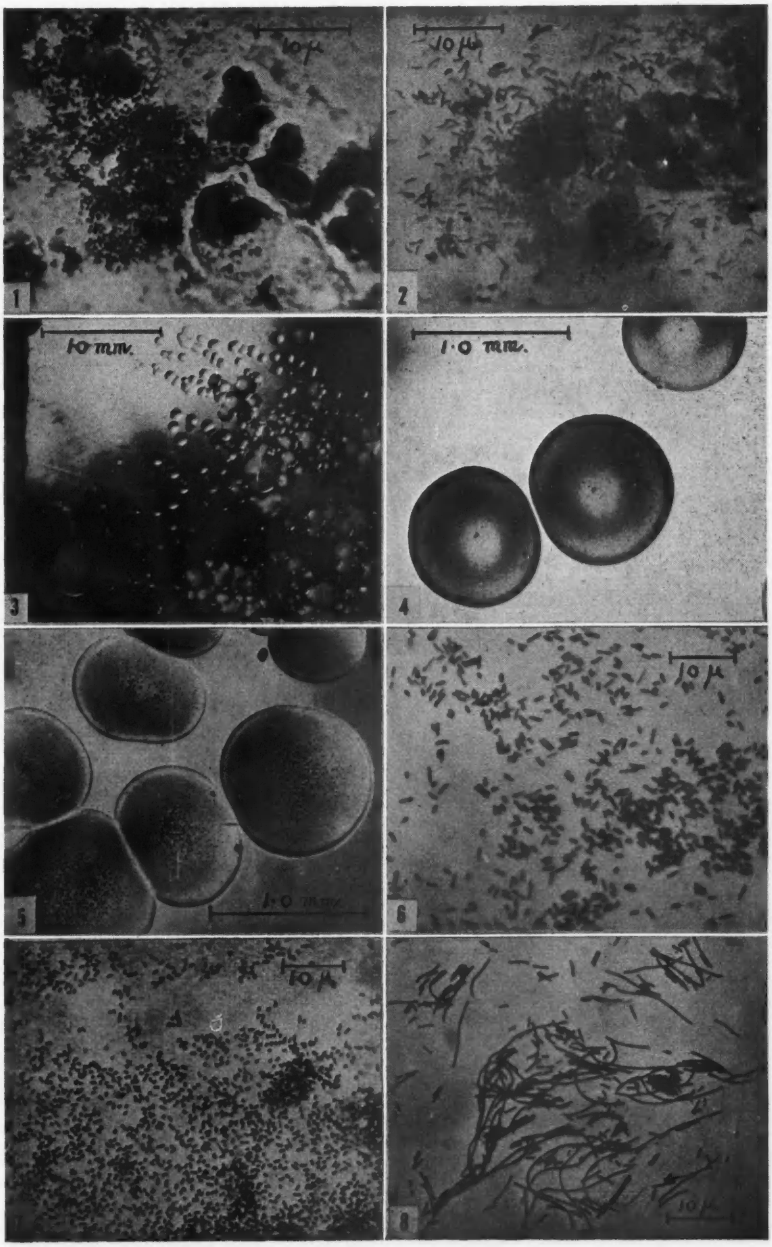
FIG. 5. Another strain of *H. vaginalis*, showing granulation in the center of the colony: 48 hours at 37°.

FIG. 6. Typical appearance of a recently isolated strain of *H. vaginalis*.

FIG. 7. Coccobacillary form of *H. vaginalis*. This strain had been maintained on chocolate agar for 6 months.

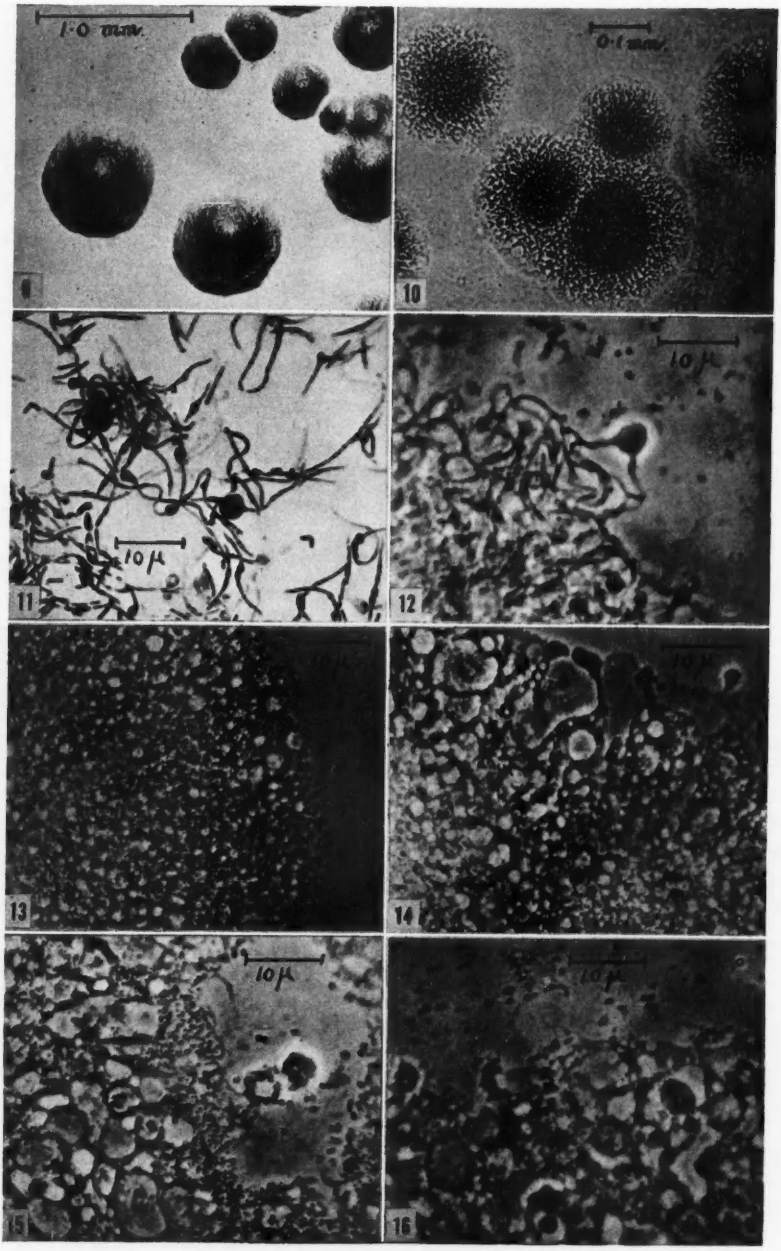
FIG. 8. *H. vaginalis*: long filamentous forms occurring as the result of spontaneous dissociation.

PLATE I



Amies and Jones—Can. J. Microbiol.

PLATE II



Amies and Jones—Can. J. Microbiol.

Novobiocin:	sensitive to 2 mcg.	14 strains
	insensitive to 2 mcg.	7 strains
Erythromycin:	sensitive to 1 mcg.	23 strains
	insensitive to 1 mcg.	0 strains

Antigenic Structure

Young adult rabbits received a preliminary intradermal injection of 100 million living cells from a 24-hour culture of the selected strain. When the local reaction had subsided the animals were hyperimmunized by giving a series of intravenous injections of the same strain. The dose was increased gradually to a maximum of 5000 million living organisms, given twice weekly. Usually the animals remained in good condition, but a few developed signs of a low grade generalized infection from which they died.

In preliminary tests a slide agglutination technique was tried and found to give satisfactory results, provided that recently isolated strains were employed: after some months of serial transplantation on culture media spontaneous agglutination interfered seriously with the interpretation of the tests. Of the 25 strains available for study, 18 on primary isolation, were specifically agglutinated by a single antiserum prepared against one member of the group. Five strains which did not react were agglutinated by a second serum prepared against one of their number. The two remaining strains were agglutinated by their own homologous antiserum, but not by any other. Twenty-six recently isolated and probably pathogenic strains of *H. influenzae* (one from the meninges and 25 from the respiratory tract) and two strains of *H. pertussis* were also tested with the *H. vaginalis* antisera. There was no cross agglutination.

An unexpected difficulty arose when attempts were later made to carry out quantitative tests, using a tube technique. Each of the 25 strains was found to flocculate spontaneously in physiological saline; and no procedure, such as reducing the salt content, using a phosphate buffer, or adding a protective colloid, was effective in preventing this. It was therefore concluded that laboratory strains soon become partly rough in character even though the microscopic and colonial appearances are those of the normal smooth phase.

This lack of suspension stability has so far prevented us from determining whether *H. vaginalis* is related antigenically to any other members of the

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FIG. 9. Rough variant colonies of *H. vaginalis*. The corresponding smear preparation is shown in FIG. 8.

FIG. 10. "Pleuropneumonia-like" colonies on serum yeast agar. First subculture from a Peizer plate inoculated with vaginal exudate.

FIG. 11. Long filaments with fusiform swellings: an early effect of penicillin on *H. vaginalis*.

FIGS. 12, 14, 15, 16. Small colonies of *H. vaginalis* growing on serum–yeast agar containing penicillin. Structure of the still-living colonies as demonstrated by phase contrast microscopy. Dark bodies, large saccular light bodies, and elementary bodies are all shown.

FIG. 13. Part of one of the PPLO colonies illustrated in FIG. 10 as seen by phase contrast microscopy. The appearance is essentially the same as the L type colony of *H. vaginalis* shown in FIGS. 14–16.

genus. In order to obtain information on this matter other serological procedures will have to be used. A haemagglutination–inhibition reaction is at present being studied.

Pathogenic Characters

The investigations have so far been limited to experiments on laboratory animals. Rabbits, guinea-pigs, white mice, and golden hamsters were inoculated with a number of the available strains, using 24-hour cultures in a dose of 100 to 1000 million living cells. The subcutaneous, intradermal, intravenous, intraperitoneal, and intracerebral routes were used. The rabbit was the only animal found to be susceptible to infection. Intradermal injection of 100 million living cells produced a circumscribed, slightly raised, red lesion measuring about 15 by 25 mm. Necrosis did not occur, but resolution was slow, taking 10 to 15 days. The histological appearance of the lesions had no distinctive characters. A single dose of approximately 100 million living organisms, given intravenously, had no effect; but in some animals repeated intravenous injections produced fever, loss of weight, and dyspnea. No evidence of localization was found at the post-mortem examination and sectioned material was not informative.

The L Forms of *Haemophilus vaginalis*

Although it is relatively insensitive to penicillin, *H. vaginalis* is affected by a high concentration of this antibiotic. Colonies bordering on an area of complete inhibition of growth are small, flat, irregular in shape, and less shiny than usual. Stained smear preparations of such colonies reveal long Gram-negative filaments with conspicuous fusiform swellings (Fig. 11). Similar changes resulting from the action of penicillin have been described in many kinds of bacteria and it is now well established that these represent an early stage of transformation to the L type of growth. Further study showed that *H. vaginalis* is readily converted into the L form. To demonstrate these changes it was necessary to examine the small colonies with the least possible manipulation. This was achieved by placing over the colony a microscope cover glass, either untreated or previously covered with a dry film of Azure II or methylene blue stain as recommended by Dienes (3). If no stain were applied, the colony remained viable and the culture plate could be re-examined after further incubation. The intact colony could thus be studied in situ with the phase microscope, using the long-focus phase condenser and corresponding objectives manufactured by Zeiss-Winkel. In this manner it was possible to follow the development of the L forms. The formation of filaments with fusiform swellings was readily seen at the edge of the small colonies (Fig. 12). Later, the filaments could no longer be seen, but the fusiform swellings had changed into large spherical or ovoid bodies which appeared dark with phase illumination. Later still, the dark bodies were converted into what appeared to be empty sacs which appeared light with phase illumination. Figs. 14 to 16 illustrate these changes. Large numbers

of very small particles could also be seen adjacent to or lying between the light bodies, but it was not possible to form a definite opinion whether these particles were derived from the large bodies. The presence of the light bodies with a minimum of darker material between them gave a foam-like appearance to the colony which is characteristic of the L transformation. The visual appearances were much more definite than the illustrations suggest: the lack of contrast in the images rendered photography difficult. This description and the accompanying illustrations will be recognized to agree closely with Dienes' numerous writings on this subject. The review article by Dienes and Weinberger (5) contains references to most of the literature on this subject.

So far we have not been able to maintain this L type of growth in serial subcultures. If the organisms were returned to the ordinary serum–yeast agar medium without penicillin the normal bacterial forms quickly reappeared: if kept on penicillin medium growth in the L phase was maintained for a while but ultimately died out.

Further experience of this method of examining plate cultures showed that very small L-type colonies could often be found on primary culture plates inoculated with vaginal exudate. These colonies, sometimes microscopic in size, were found in the area bordering on the zone of penicillin diffusion (Figs. 10, 13). They could be maintained, though with difficulty, in serial subculture by using the agar block method of transplantation (12). Reversion to a normal bacterial morphology did not occur. These findings recalled the well-known work of Dienes and many others (4, 12, 16, 18, 19) on the presence of pleuropneumonia-like organisms (PPLO) in genitourinary infections. The relationship of penicillin-induced L forms and spontaneously occurring PPLO will be discussed later.

Discussion

The frequent occurrence of very small Gram-negative rods and coccobacilli in vaginal smears must be familiar to all who are engaged in routine medical bacteriology. It is surprising, therefore, that the nature of these organisms was not discovered long ago. Failure to use a suitable medium does not seem an adequate explanation because rich media containing serum or ascitic fluid have long been employed for the study of PPLO in genitourinary disease. It is difficult to believe that workers in this field ignored all organisms that did not produce an L-type of colony and so failed to recognize the normal bacterial type. The more likely explanation is that all small translucent colonies which developed on the plate cultures were regarded as fecal streptococci. Those who attempt to confirm the findings of Gardner and Dukes will almost certainly encounter this difficulty. Examination of stained smears is the only certain method of differentiation. The specimens available for our studies did not yield very large numbers of *H. vaginalis* colonies; and we have never seen a plate culture "covered with minute pin-point colonies" which proved to be this organism.

We were not able to make an exhaustive search of the older literature, but it is evident that within recent years little attention has been given to this subject. A few earlier reports may perhaps be regarded as the prelude to the work of Gardner and Dukes. In 1947 Henriksen (10) described a study of Gram-negative bacilli of the genitourinary tract. In this paper he mentioned that he had isolated five strains of an organism indistinguishable from *H. influenzae*. No detailed description of these strains was given. The isolation of one strain of *H. influenzae* from the female genital tract is also reported in an earlier communication by Thompson (21). The organism described by Leopold (15) as a common inhabitant of the chronically infected genitourinary tract differs in several important respects from *H. vaginalis*. It is haemolytic, definitely micro-aerophilic, grows in plain thioglycollate broth and does not reduce nitrates.

The pathogenicity of *H. vaginalis* seems to vary considerably. Gardner and Dukes' experiments on human volunteers show that a clinically recognizable infection is not easily obtained. On the other hand, it may be the only demonstrable organism in a severe vaginitis with a markedly purulent exudate. The possibility that the organism is merely playing a secondary role in such cases cannot be excluded, but this seems unlikely. Different strains may vary widely in pathogenicity, or the virulence of a single strain may be altered by factors not yet understood. In addition, other factors may operate on the host rather than on the bacterial cell. Physiological activity and chemical irritants have a profound effect upon the susceptibility of the genital mucous membrane.

In some respects there is a striking similarity between *H. vaginalis* and the Koch-Weeks bacillus (*H. aegyptius*). Both organisms, when they are producing an acute infection, appear as small extremely thin rods lying between or within pus cells. As the infection subsides, however, the bacteria assume the more normal appearance of small pleomorphic cells, often in the form of coccobacilli or diplobacilli. Pus cells are no longer numerous and the bacteria are often found attached to the surface of flattened epithelial cells. This change in morphology with decrease of virulence was noted by one of us (C.R.A.) in Egypt. During the hot months of the year acute Koch-Weeks conjunctivitis was frequently seen. As the cold season approached and the incidence of ophthalmia decreased, there was a gradual transition from the small thin to the small pleomorphic type of organism in conjunctival smears. Study of vaginal smears, in the light of this previous experience, has suggested that morphology and virulence may be similarly correlated in the case of *H. vaginalis*.

We return now to the problem raised by the finding of what appeared to be L-phase colonies in the primary culture plates. Can it be inferred from our experiments that *H. vaginalis* is the probable origin of the organisms hitherto described as PPLO of the genitourinary tract ? This raises the more general question of whether PPLO are stable L forms of bacteria or a special class of microbe not related in any way to bacteria. Klieneberger (13) with great

experience of both L forms and true PPLO insists that the two are entirely distinct. This is also the opinion of Edward (6, 7) and Freundt (7, 8). If this is so, our experiments must be interpreted as evidence that some of our primary culture plates contained both PPLO and *H. vaginalis*, the latter being subsequently converted by means of penicillin into the L type of growth. We are not able to differentiate between these two kinds of colony on morphological grounds: the authors that are cited above claim that they can do so.

In the light of this new evidence we think it is reasonable to suggest that in most cases organisms described by other workers as PPLO of the genitourinary tract were, in fact, L forms of *H. vaginalis*. This would provide a possible explanation of why vaginal exudates which, according to stained smears, contain the organism in large numbers only yield on culture a slight growth of typical *H. vaginalis* colonies. The majority have undergone L-phase variation and therefore grow with difficulty. The essential similarity of PPLO and L colonies seems to be accepted by some writers (16, 18). Dienes and Weinberger (5) adopt a more cautious attitude, pointing out that "the pleuropneumonia group is in the same position as the *Fungi imperfecti*; they form a morphological group of miscellaneous origin, the distribution of which in the natural system is at present impossible."

If it is finally proved that the genitourinary PPLO are actually L forms of *H. vaginalis*, the whole subject of "non-specific" infections in this area of the body will be clarified. Attention could then be turned from these non-pathogenic variants to the normal pathogenic cultivable form. Perhaps the time is not far off when the diagnosis of *H. vaginalis* infections by smear and culture is a routine procedure.

Addendum

Since the completion of this report three short papers relevant to the subject have come to our attention. Ray and Maughan*(64 : 581–587, 1956) have been able to confirm the clinical findings of Gardner and Dukes. Deming† has suggested that *H. vaginalis* is the same as *H. influenzae*. Our own work lends no support to this belief. Finally, Lutz, Grooten, and Wurch‡ have described an organism, frequently present in vaginal exudates, to which they have given the name *Haemophilus hemolyticus vaginalis*.

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SOME RELATIONSHIPS OF THE SOMATIC ANTIGENS OF A GROUP OF SERRATIA MARCESCENS CULTURES[1]

BETTY R. DAVIS[2] AND JOHN M. WOODWARD

Abstract

Using heated antigens, antisera were prepared against the somatic antigens of 16 strains of *Serratia marcescens*. By means of cross-agglutination tests it was possible to divide the strains into six groups. Groups II and VI each contained mutually adsorptive strains, which indicated that the members within respective groups are closely related. No correlation was observed of serological activity and pigment production among the pigmented and non-pigmented strains.

Introduction

Reports concerning the serological characteristics of the *Serratia* have been few. Schad (4), using *Serratia marcescens* (*B. prodigiosus*) to test the efficiency of filtration through the soil, employed specific agglutination as the means for identifying the strains recovered. He tested 42 cultures with sera prepared against the München and Kiel strains of *S. marcescens*. With this exception there is no information available concerning the serological grouping of these organisms.

This investigation was conducted to determine the serological relationships among 16 strains of *S. marcescens*. The study was restricted to the somatic antigens and no attempt was made to characterize the flagellar components.

Materials and Methods

Cultures

Sixteen cultures of *S. marcescens*, including both pigmented and non-pigmented strains, were employed. The sources and pigmentation of the strains are given in Table I. They possessed the following biochemical characteristics: Acid but no gas was produced from glucose, glycerol, lactose, maltose, salicin, and xylose. Urease, indole, and methyl red tests were negative. They were citrate positive, Voges-Proskauer positive, liquefied gelatin, reduced nitrate, and were motile. The cultures were grown and maintained on nutrient agar.

Preparation of Serum for Somatic Antigens

Antisera were prepared by injecting rabbits with boiled broth cultures of each of the 16 strains, as described by Edwards and Bruner (1) in their studies of the *Salmonella* group. All animals were tested prior to immunization to determine that they possessed no agglutinins for *Serratia*.

[1]Manuscript received February 5, 1957.
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TABLE I

SOURCE AND PIGMENTATION OF STRAINS OF *Serratia marcescens*

Strain	Source	Pigment*
U.T.	Unknown	+
15	Abdominal incision	0
219	Throat	0
220	Throat	0
232	Abscess	0
293	Bronchial	0
533	Urine	0
555	Kidney	+
808	Urine	+
840	Urine	+
1155	Bronchial	0
1176	Bronchial	0
1277	Spinal fluid	0
1414	Flies	+
1507	Unknown	0
1698	Throat	+

*Trypticase soy agar after 48 hours at room temperature.

Agglutination Tests

Antigens for agglutination tests were prepared by heating 24 hour broth cultures at 100° C. for 2 hours. Cross-agglutination tests were conducted with all strains and sera to determine possible groupings. Subsequently, reciprocal agglutinin adsorption tests were performed according to the methods of Edwards and Ewing (3) to study antigenic relationships among members of the various groups so established.

Results

Agglutination Tests

Tube agglutination tests, the results of which are given in Table II, indicated that the cultures could be divided into six groups, between which there were only a few minor relationships. Strain 232 possessed distinct O antigens and reacted only slightly with sera for members of the other five groups, and antigens prepared with members of the other groups gave little or no reaction with antiserum for 232. In the second group consisting of strains 533 and 1507 each culture agglutinated to the homologous titer of antiserum prepared with the other. The third, fourth, fifth, and sixth groups contained three, two, five, and three strains, respectively. Some cross-reaction was observed between groups III and IV but little or none was detected among the others.

Adsorption Tests

These tests demonstrated that group II and group VI each were homogeneous since each component strain of the respective groups removed all agglutinins from sera for all other members of the group. That is, they were mutually adsorptive and represented a single antigenic type.

TABLE II

AGGLUTINATION TESTS

Group	Serum	Antigen															
		232	533	1507	15	220	1155	219	1176	293	555	808	840	1414	U.T.	1277	1698
I	232	*2560**	0	0	0	0	0	10	10	10	10	0	0	0	0	0	0
II	533	20	*1280*	2560	20	0	0	0	0	0	20	5	0	0	0	0	0
	1507	0	1280	*1280*	0	0	0	0	0	0	0	0	0	0	0	0	0
III	15	10	0	0	*640*	640	320	20	40	0	0	0	0	0	0	0	0
	220	0	0	0	1280	*1280*	640	80	80	0	0	0	0	0	0	0	0
	1155	0	0	0	640	1280	*640*	0	0	0	0	0	0	0	0	0	0
IV	219	0	0	0	40	40	40	*1280*	640	0	0	0	0	0	0	0	0
	1176	0	0	0	20	10	10	640	*1280*	0	0	0	0	0	0	0	0
V	293	0	0	0	0	0	0	0	0	*640*	320	160	160	160	0	0	0
	555	0	0	0	0	0	0	0	0	320	*1280*	320	160	160	0	0	0
	808	0	0	0	0	0	0	0	0	640	1280	*1280*	320	320	0	0	0
	840	0	0	0	0	0	0	0	0	320	160	160	*320*	80	0	0	0
	1414	0	0	0	0	0	0	0	0	320	640	320	160	*320*			
VI	U.T.	0	0	0	0	0	0	0	0	0	0	0	0	0	*640*	160	160
	1277	0	0	0	0	0	0	0	0	0	0	0	0	0	1280	*5160*	1280
	1698	0	0	0	0	0	0	0	0	0	0	0	0	0	40	320	*320*

*Numbers indicate the reciprocal of the highest dilution giving agglutination. 0 indicates no agglutination at a dilution of 1–10.

Cross-agglutination tests between groups III and IV indicated a minor relationship between the two. In group III, strains 15 and 1155 were mutually adsorptive and agglutinins in sera for strains 15 and 1155 were removed completely by adsorption with culture 220 (Table III). The failure of strains 15 and 1155 to remove all agglutinins from the 220 serum indicated the presence of a minor antigen in strain 220 which was absent in the other two. Adsorption of the 220 serum with strain 219 removed agglutinins for members of group IV but the homologous titer was not reduced.

The adsorption of 219 serum with strain 1176 removed agglutinins for 219 and 1176 although low titer agglutinins remained for strain 220. Adsorption of 1176 serum with strain 219, without removal of all the agglutinins, indicated the presence of a minor antigen in 1176 which was not shared by 219.

Within group V, adsorption of 293 serum with strains 555, 808, and 1414, respectively, removed all of the agglutinin from the serum in each instance (Table IV). Adsorption of 293 serum with strain 840 removed all antibodies

TABLE III

ADSORPTION TESTS WITH *Serratia marcescens* GROUPS III AND IV

		Antigen					
		Group III				Group IV	
	Serum	15	220	232	1155	219	1176
Group III	15						
	adsorbed by 220	0*	0	0	0	0	0
	1155	0	0	0	0	0	0
	220						
	adsorbed by 15	0	10		0	0	0
	1155	0	10		0	40	0
	219	1280	1280		1280	0	0
	1176	320	320		320	0	0
	1155						
	adsorbed by 15	0	0		0		
	220	0	0		0		
Group IV	219						
	adsorbed by 1176	0	10		0	0	0
	1176						
	adsorbed by 219	40	0		20	0	40

*Numbers indicate the reciprocal of the highest dilution giving agglutination. 0 indicates no agglutination at a dilution of 1–10.

TABLE IV

ADSORPTION TESTS WITH *Serratia marcescens* GROUP V

Serum	Antigen					Serum	Antigen				
	293	840	555	808	1414		293	840	555	808	1414
293						808					
adsorbed by						adsorbed by					
840	20*	0	0	0	0	293	0	0	80	20	10
555	0	0	0	0	0	840	0	0	40	40	10
808	0	0	0	0	0	555	0	40	0	0	0
1414	0	0	0	0	0	1414	0	0	0	0	0
840						1414					
adsorbed by						adsorbed by					
293	0	0	0	0	0	293	0	20	80	80	20
555	0	0	0	0	0	840	0	0	160	320	160
808	0	0	0	0	0	555	0	20	0	0	0
1414	0	40	0	0	0	808	0	40	0	0	0
555						555					
adsorbed by						adsorbed by					
293	0	0	40	10	0	808	0	20	0	0	0
840	0	0	80	20	10	1414	0	20	0	0	0

*Numbers indicate the reciprocal of the highest dilution giving agglutination. 0 indicates no agglutination at a dilution of 1–10.

for strains 555, 808, 840, and 1414 but left a minor factor for culture 293. Adsorption of the 840 serum with strains 293, 555, or 808 resulted in complete removal of all agglutinins. Agglutinins for all members except the adsorbing strain remained in antiserum 1414 following adsorption by culture 293.

Adsorption of 555 or 808 serum with strain 293 and 840 removed agglutinins for both and reduced the titer for the remaining members of group V. Agglutinins for all members except the adsorbing strain remained in antiserum 1414 following adsorption by culture 293. Adsorption of this serum with strain 840 resulted in a serum that gave no reaction with 293 and 840 but which reacted with 555, 808, and 1414.

The adsorption of 555, 808, or 1414 sera with strains 555, 808, or 1414 removed agglutinins for all three strains and for 293 as well.

In some instances, i.e., adsorption of 555 serum with strains 808 and 1414, 808 serum with strains 555 and 1414, and 1414 serum with strains 555 and 808, the resulting sera agglutinated culture 840 but not the homologous strains. A similar observation has been reported by Edwards and Bruner (2) in a study of the antigenic behavior of *Salmonella pullorum*. They attributed this response to an antigen in the homologous strain, present in quantities sufficient to produce low titer agglutinins, but not present in sufficient amount to produce agglutination. This may serve to explain the circumstances which prevail in this instance.

Discussion

The separation of the 16 strains of *S. marcescens* into six serological groups indicates the possibility that such a method may be extended to other cultures of this species. For convenience, the groups discussed here have been designated by Roman numerals I to VI. Since a small number of cultures was employed it is quite probable that many additional groups may exist among other strains. Consequently, these designations are not intended to serve any purpose other than to identify the cultures used in this study.

Although some cross agglutination was observed, notably between strains of groups III and IV, the separation into six groups was quite distinct. Differences among strains within the individual groups was apparent in some instances as determined by adsorption tests. These differences, however, were minor as indicated by the low titer of the agglutinins remaining in the sera after adsorption.

It is of interest to note that all of the pigmented strains fall into two groups. Four of the five members of group V and two of the three strains of group VI are pigmented. Schad (4) in testing cultures recovered from water found that both pigmented and non-pigmented strains reacted with the *S. marcescens* sera he used. Considering the variation in pigment production to which these organisms are subject, and the small number of strains represented here, it would be difficult to draw conclusions concerning any parallelism between pigmentation and serological relationships.

This analysis is incomplete since no attempt has been made to determine the presence of K antigens or to identify the H antigens. Such identification would be necessary before such strains as 533 and 1507 or U. T., 1277, and 1698 could be said to be either antigenically identical or different types within the same O groups.

Acknowledgment

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ALCOHOLIC FERMENTATION UNDER AERATION[1]

L. A. CAMPBELL and S. S. HEGBOM

Abstract

Saccharomyces fragilis was grown at oxygen absorption rates of 0.4, 1, 3, and 5.2 g. per liter per hour both at 20° C. and at 37° C. A high rate of yeast production as well as alcohol formation was found at 37° and all four oxygen absorption rates. At 20° alcohol was formed only at the two lowest rates of oxygen absorption. An exponential relation between the maximum yeast population density possible without alcohol production and the rate of oxygen absorption was derived.

Introduction

Lemoigne, Aubert, and Millet (6) have shown that *Saccharomyces cerevisiae* produces ethanol under conditions which they consider aerobic. This appears to be an instance when the Pasteur effect does not operate. The process has been elucidated by Aubert and Milhaud (1, 7, 8), and these two authors have also demonstrated the reversibility of glycolysis (2). Blumenthal, Lewis, and Weinhouse (3) have attempted to estimate the relative participation of the Embden–Meyerhof and the shunt pathways in glucose catabolism in several yeasts under anaerobic and aerobic conditions. Our work, undertaken in connection with the production of protein by *Saccharomyces fragilis* on cheese whey, has shown that fermentation temperature is important in determining whether or not the Pasteur effect is observed and that much caution must be used in deciding whether or not experimental conditions are aerobic.

Materials and Methods

Equipment

The tank used was stainless steel of 20 gal. capacity fitted with: a stirring propeller the speed of which could be varied, a porous ceramic disk air sparger, a stainless steel attemperating coil, and a sampling valve. The air supply was passed through a humidifying column, then through two closely-packed glass wool filters which were dry-heat sterilized. The rate of flow into the tank was measured by a Fischer and Porter Flowrator. The pH was controlled at 4.7 by a Beckman titrator feeding 10% aqueous ammonia. The temperature was thermostatically controlled by passage of either hot or cold water through the attemperating coils.

Growth Conditions

The yeast used was *Saccharomyces fragilis* A.T.C.C. No. 8567. The seed-yeast was developed anaerobically at 37° through three stages: 70 ml. (48 hours) into 700 ml. (24 hours) into 7000 ml. (24 hours). Each stage of the medium was divided into two equal portions and consisted of: crude

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lactose 5.0%, KH_2PO_4 0.5%, K_2HPO_4 0.25%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.75% (sterilized separately). This seed-yeast was then transferred to the tank containing 72 liters of the following growth medium: crude lactose 2.35%, KH_2PO_4 0.1%, K_2HPO_4 0.05%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.65% (sterilized separately). Foam was controlled by the addition of 100 ml. of lard oil.

The yeast was grown at two temperatures, each at four separate rates of oxygen absorption. The rates of oxygen absorption were varied by changing either the air flow or the turbulence as controlled by the propeller speed. Yeast, ethanol, and lactose were measured throughout the course of each experiment.

Analytical Methods

Yeast weight: 100 ml. medium was centrifuged, the supernatant poured off, and the precipitated yeast washed by resuspension in distilled water and again recovered centrifugally. This was transferred to tared aluminum dishes, and weighed after drying at 95°.

Ethanol: 2 ml. supernatant from yeast weight determination was added to 8 ml. water in a round bottom distilling flask connected with a Liebig condenser. Immediately before the start of distillation 20 ml. potassium dichromate solution (4.26 g. per liter) were mixed with 10 ml. conc. sulphuric acid and this hot liquid placed so that the top of the adapter was just submerged below the surface. About 5–7 ml. were then distilled over, and the residual dichromate measured by titration with ferrous ammonium sulphate (92 g. + 12 ml. conc. sulphuric acid made up to 1 liter). One milliliter of a 0.4% solution of potassium ferricyanide was used as indicator. The amount of ethanol was calculated after reference to a standard graph, previously prepared.

Lactose was determined by the method of Somogyi (5).

Rate of oxygen absorption from aqueous sodium sulphite solutions in the equipment was determined by the method of Cooper *et al.* (4), for different degrees of turbulence. Turbulence was varied by changing either the air flow rate or the propeller speed, or both.

Results

Rates of Oxygen Absorption

The rates of oxygen absorption by aqueous sodium sulphite solution in our equipment are shown in Table I. The data show that, other variables being held constant, raising the temperature from 20° to 37° decreases the

TABLE I

RATES OF OXYGEN ABSORPTION FROM AQUEOUS SODIUM SULPHITE SOLUTION

Air flow (cu. ft./min.)	0.667	0.667	0.667	3.33	3.33	3.33	3.33
Stirrer speed (r.p.m.)	72	290	290	290	290	580	580
Gauge pressure (lb./sq. in.)	20	20	20	20	20	20	20
Temperature (° C.)	20	20	37	20	37	20	37
Oxygen absorption (g./liter/hr.)	0.40	1.09	0.99	3.03	2.93	5.22	5.16

TABLE II

EFFECT OF OXYGEN ABSORPTION RATE ON YEAST AND ETHANOL PRODUCTION AT 20°

	0.40 g. oxygen/liter/hour			1.09 g. oxygen/liter/hour			3.03 g. oxygen/liter/hour		
Hours	Yeast, g./100 ml.	Ethanol, g./100 ml.	Lactose, g./100 ml.	Yeast, g./100 ml.	Ethanol, g./100 ml.	Lactose, g./100 ml.	Yeast, g./100 ml.	Ethanol, g./100 ml.	Lactose, g./100 ml.
0	0.047	0.20	2.14	0.037	0.03	2.25	0.032	0.10	2.28
4				0.018	0.03	2.23	0.221	0.10	2.27
6				0.028	0.02	2.22			
8	0.039	0.20	2.12	0.046	0.03	2.20			
10	0.045	0.18	2.04						
12	0.058	0.17	2.02	0.087	0.03	2.15			
13	0.078	0.17	1.95				0.056	0.10	2.20
14							0.079	0.10	2.18
16	0.098	0.16	1.90	0.153	0.03	2.08			
17	0.107	0.20	1.73				0.115	0.10	2.14
18				0.215	0.02	1.95			
19	0.129	0.23	1.66				0.164	0.07	2.10
20				0.298	0.01	1.80			
21							0.234	0.05	2.00
22				0.379	0.01	1.67	0.280	0.04	1.95
24				0.471	0.06	1.48	0.401	0.03	1.83
26				0.561	0.11	1.04	0.545	0.02	1.50
28				0.664	0.19	0.64	0.651	0.03	1.25
30				0.750	0.29	0.10	0.760	0.02	0.80
32				0.794	0.38	0.00	0.868	0.02	0.46
34				0.846	0.29		1.065	0.01	0.22
36				0.889	0.20		1.100	0.00	0.00
38				0.935	0.11				
40				0.933	0.03				

TABLE III

EFFECT OF OXYGEN ABSORPTION RATE ON YEAST AND ETHANOL PRODUCTION AT 37°

Hours	0.40 g. oxygen/liter/hour			0.99 g. oxygen/liter/hour			2.93 g. oxygen/liter/hour		
	Yeast, g./100 ml.	Ethanol, g./100 ml.	Lactose, g./100 ml.	Yeast, g./100 ml.	Ethanol, g./100 ml.	Lactose, g./100 ml.	Yeast, g./100 ml.	Ethanol, g./100 ml.	Lactose, g./100 ml.
0	0.032	0.11	2.35	0.026	0.07	2.43	0.032	0.08	2.20
1	0.046	0.11	2.25	0.021	0.07	2.42	0.030	0.08	2.16
2	0.045	0.11	2.25	0.029	0.07	2.41	0.039	0.08	2.10
3	0.062	0.11	2.20	0.041	0.07	2.35	0.060	0.03	2.00
4	0.096	0.15	2.20	0.065	0.07	2.28	0.095	0.03	1.85
5	0.133	0.18	1.88	0.101	0.11	2.20	0.167	0.10	1.65
6	0.174	0.28	1.60	0.152	0.21	1.52	0.272	0.17	1.25
7	0.226	0.32	1.10	0.203	0.36	0.80	0.383	0.27	0.50
8				0.249	0.54	0.32	0.479	0.32	0.00
9				0.308	0.76	0.00	0.562	0.24	
10				0.348	0.73		0.602	0.19	
11				0.376	0.72		0.767	0.00	
12				0.401	0.68				

rate of oxygen absorption by only 0.1 g. per liter per hour. Although these results are for aqueous sodium sulphite solution, it is assumed that with the medium used, the rates of oxygen absorption will be relatively the same for the same propeller speeds and air flow rates.

Yeast Metabolism

The results of the experiments on yeast growth and ethanol production at rates of oxygen absorption of 0.4, 1, and 3 g. per liter per hour at 20° are shown in Table II. The results for the same oxygen absorption rates at 37° are shown in Table III. For each oxygen absorption rate at 37° there is a point during the growth of the yeast at which the ethanol content of the medium begins to rise. This is also the case at 20° and an oxygen absorption rate of 1 g. per liter per hour. However, at 20° and an oxygen absorption rate of 3 g. per liter per hour, ethanol does not accumulate but continues to disappear throughout the experiment. At an oxygen absorption rate of 0.4 g. per liter per hour the ethanol content does not vary.

At the still higher rate of oxygen absorption of 5.2 g. per liter per hour the contrast in alcohol metabolism between 20° and 37° is even more marked, and is shown graphically in Fig. 1.

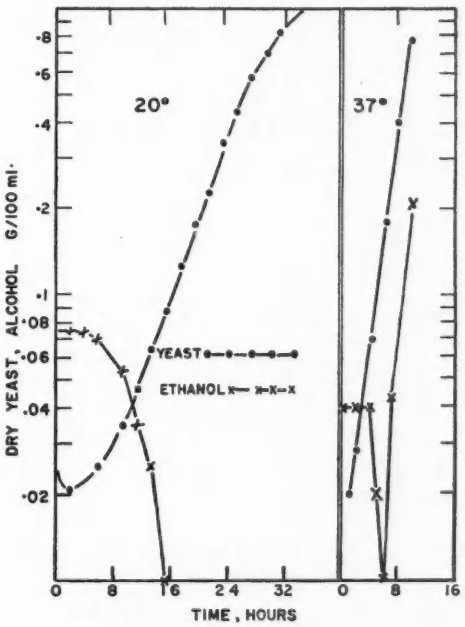

FIG. 1. The influence of temperature on the production of yeast and ethanol at an oxygenation rate of 5.2 g. oxygen per liter per hour.

Discussion

Examination of Fig. 1 shows that at 20° the ethanol which was introduced along with the seed-yeast was gradually used up along with the sugar as the yeast grew. This is a typical illustration of the Pasteur effect. In the 37° experiment however, this was the case for the first 6 hours only, after which ethanol as well as yeast was produced. From the 7th to the 10th hour, the production of alcohol was at the same rate as the production of yeast matter. This is similar to the picture shown by Lemoigne *et al.* (6). Although not shown in Fig. 1, it may be further noted that after the sugar had all been used up, the concentration of ethanol fell to a low value.

It is apparent from these results that the validity of the Pasteur effect is not in question. In those cases when it appears not to hold, this is because the rate of oxygen absorption is inadequate for the yeast population although the aeration may appear to be violent. The effect of an increase in temperature is not to decrease the rate of oxygen absorption into the liquid but to increase the rate of yeast metabolism so much that not only is new yeast formed as rapidly as conditions permit but also alcoholic fermentation takes place simultaneously.

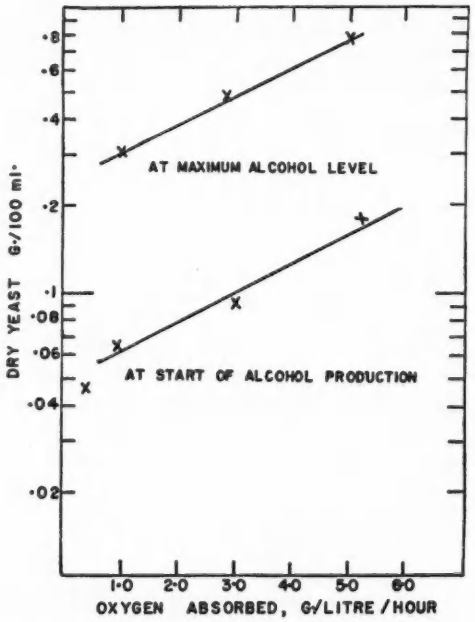

FIG. 2. The influence of the rate of oxygenation at 37° on the density of yeast population attained when ethanol production starts and when maximum ethanol production is reached.

It is to be expected from these results that at any one temperature there would be a relation between the maximum yeast population which can exist without the production of alcohol, and the rate of oxygen absorption into the medium. This relation is shown in Fig. 2 for 37°. It is of the form:

$$y = ae^{xk},$$

where y = maximum yeast population density before alcoholic fermentation begins,
x = rate of oxygen absorption into the medium,
k = a function of temperature,
a = a constant.

As a result of our work, we are unable to agree with Lemoigne *et al.* (6), who state that: "ou les conditions d'oxygenation sont bien assurées, l'aeration seule ne peut pas empêcher la formation d'alcool par la levure de boulangerie en voie de proliferation". On the contrary, our work shows that where oxygenation is adequate, alcohol is consumed and not produced. We believe that their conclusions would have been different if they had experimented at a lower temperature, or at a lower yeast level, or at a lower initial sugar level. If any or all of these three variables had been sufficiently lower, the rate of oxygen absorption with their aeration system would have been sufficient to supply truly aerobic conditions, and alcohol would not have been formed. We would suggest, too, that in the experiments reported by Blumenthal *et al.* (3) the conditions which they consider aerobic were not truly so, since they state that ethanol was "always produced in isolatable quantities"

Acknowledgment

The authors wish to acknowledge the assistance and encouragement of Dr. H. B. Speakman and Mr. W. C. Henry.

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THE PATHOGENICITY OF DEMATIUM NIGRUM FOR MICE[1]

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Abstract

Dematium nigrum, a common laboratory contaminant, was found to have an unsuspected ability to cause a neurological syndrome in mice. Intravenous injection of a suspension caused a fatal, right-sided hemiparesis. Death occurred, most often, after 6–10 days. The organism could be recovered from the brains of afflicted animals but not from other organs.

Introduction

Dematium nigrum, a dark pigmented, yeastlike fungus is a relatively common laboratory contaminant. Although widely distributed in nature, it has never been extensively studied.

During a comparative study of various *black yeasts*, its apparent pathogenicity for mice was disclosed. This report represents the investigation of this phenomenon.

Materials and Methods

Three strains of *Dematium nigrum* were generously supplied by Dr. L. J. Wickerham*: NRRL Y-279, isolated from normal skin (4); Y-322, isolated from air; and Y-652, isolated from yeast cake. Another organism, isolated from baker's yeast by Dr. E. McCoy† and identified as *Dematium nigrum* in this laboratory, was designated as A-18. *Pullularia pullulans*, another common laboratory contaminant, and *Cryptococcus nigricans* nov. sp. (8), were found to be non-pathogenic and employed as negative controls.

The organisms identified as *Dematium nigrum* exhibited the following distinguishing characteristics: dark mycelium distinct from exogenous, catenate conidia which were borne on very short, lateral conidiophores.

Certain workers place organisms having these characteristics into the genus *Phialophora*, considering *Dematium* a *nomen dubium*.‡ It is the opinion of this investigator that the existing differences between *Dematium* and *Phialphora*, i.e. exogenous vs. endogenous, and catenate vs. capitate arrangement of the conidia, are sufficient to justify the separation of these genera, as recognized by Clements and Shear (3) and by Bessey (2).

The organisms were cultured on slants of Sabouraud-dextrose agar at 28° C. for 4 days. At the end of this period the growth was scraped aseptically

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‡Opinion from Dr. G. A. de Vries (Centraalbureau voor Schimmelcultures, Baarn, Holland) to Dr. Fritz Blank of McGill University and communicated via the Editor.

from the suface of the slants and washed three times with sterile saline. Saline suspensions of each organism were standardized turbidimetrically in a Spectronic 20 Colorimeter (transmitting at 425 millmicrons) to give a turbidity equal to that of a suspension of *Cryptococcus nigricans* containing 10^4 cells/ml. The viability of the suspensions as well as their freedom from contamination were confirmed by the plating of aliquots, immediately before and after injection, on Sabouraud-dextrose and brain–heart infusion agar.

One-half milliliter of each suspension was injected into the tail veins of 20–25 g. mice. The animals were observed twice daily for the appearance of neurological symptoms. Animals exhibiting severe neurological symptoms were killed and their brains aseptically removed. Before inoculation into Sabouraud-dextrose and brain–heart infusion broth, portions of the brain were macerated and examined microscopically. The broth cultures were incubated at 28° C. and 37° C. for 48 hours after which aliquots were streaked out on agar plates. These, in turn, were incubated for 4 days and examined for the presence of *Dematium nigrum* or the other organism injected.

Suspensions of the organisms re-isolated in this manner (Y-279, Y-650, and A-18) were injected into another group of animals. These were killed when neurological symptoms appeared. Lung, kidney, spleen, blood, liver, and brain were removed, aseptically macerated, and cultured as above.

The time–effect (fatality) curves for the virulent organisms were calculated and plotted according to the graphic method of Litchfield (6, 7).

Results and Discussion

As can be seen in Table I, the injection of Y-279, Y-652, A-18, and, to a lesser extent, Y-322 produced what appeared to be a neurological syndrome, followed usually by death. The symptoms may be described as follows.

The first suggestion of abnormality was a permanent tilting of the head to the right. The animals appeared to be otherwise normal during this stage, which lasted from 6 to as long as 18 hours. This was followed by a paresis of the right foreleg resulting in an ever increasing tendency to circle to the right. As this progressed, the animals exhibited signs of general irritability, e.g. ruffled fur and increased sensitivity to touch. The paresis of the right

TABLE I

INCIDENCE OF NEUROLOGICAL SYNDROME AND DEATH IN ANIMALS INJECTED WITH THE ORGANISMS UNDER INVESTIGATION

Organism	No. animals injected	Neurological syndrome	Deaths	50% fatality (days)
Cryptococcus nigricans	10	0	0	—
Pullularia pullulans	9	0	0	—
Dematium nigrum (Y-279)	10	3	7	9.5
Dematium nigrum (Y-322)	10	2	1	—
Dematium nigrum (Y-652)	10	8	7	6.7
Dematium nigrum (A-18)	10	6	7	9.5

foreleg was soon accompanied by a general incapacitation of the entire right side. This inhibited circling to the right, although the application of tactile stimulation still resulted in attempted movements to the right. Animals exhibiting the more severe symptoms invariably died.

The microbiological flora of animals exhibiting this syndrome was determined by culture of the aseptically removed organs. It is interesting to note that the yeastlike fungi could only be recovered from the brain of animals exhibiting the above syndrome and could not be isolated from any of the other organs of the same animals (Table II). The sporadic occurrence of other microorganisms in the tissues of the animals injected with the virulent yeastlike fungi did not differ from the pattern observed for the controls, i.e. those injected with saline, *Pullularia pullulans*, and *Cryptococcus nigricans*, and was not considered significant. The pathology of the brain was not studied.

The lines which represent the time–effect curves (Fig. 1) for each of the virulent organisms were determined to be statistically alike with respect to both slope and confidence limits (95% probability). Using the same criteria, the lone fatality in the group injected with Y-322, although accompanied by the neurological syndrome, could not be considered statistically significant.

The observation that the neurological syndrome manifested itself primarily on the right side initially suggested that the phenomenon was the result of a non-specific, physical occlusion of the neurovascular system. However, the lack of response to the injection of the other organisms employed appeared to invalidate this hypothesis. Further, it is felt that the observation of budding cells (leading to the formation of pseudomycelia) in the macerated brain of afflicted mice points toward a specific infection of the nervous tissue.

TABLE II

MICROBIOLOGICAL FLORA OF ANIMALS INJECTED WITH THE ORGANISMS UNDER INVESTIGATION

Organs	Animals injected with:																				
	Saline					*Cryptococcus nigricans*					*Dematium nigrum* Y-279					*Dematium nigrum* A-18					
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	
Liver					d																
Spleen			R				d r					d		R	d			d d			
Blood																					
Kidney				R	d	d	R						d					d d			
Brain											B	B	B	B	B	B	B	B	B	B	

NOTE: Key to organisms isolated:
R = Gram-negative rods.
r = Gram-positive rods.
d = Gram-positive diplococci.
B = Black, yeastlike fungi.

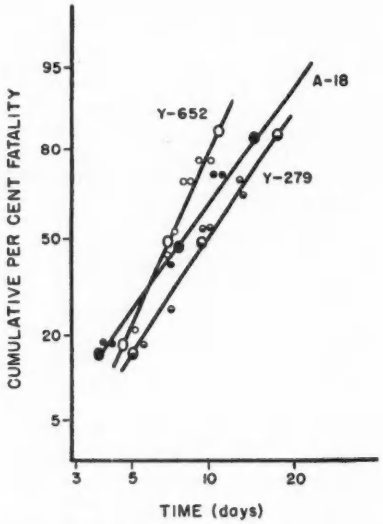

FIG. 1. Time–effect curve for fatalities resulting from the injection of *Dematium nigrum* (Y-279, Y-652, and A-18).

Because three of the four strains of *Dematium nigrum* tested (the fourth elicited a similar but reduced response) caused nervous tissue involvement accompanied by death when injected into mice, there would appear to be a need for care in handling this laboratory contaminant. This is further emphasized by the correlation between mouse virulence and human pathogenicity which has been demonstrated for other yeastlike fungi (5). Indeed, mouse virulence accompanied by brain involvement, is one of the few characteristics used to differentiate the pathogenic from the non-pathogenic cryptococci (1).

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A DISEASE OF GRASSHOPPERS CAUSED BY THE BACTERIUM PSEUDOMONAS AERUGINOSA (SCHROETER) MIGULA[1]

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Abstract

Pseudomonas aeruginosa is the chief cause of disease in laboratory cultures of grasshoppers. A large number of strains of the bacterium from different localities fell into five types on the basis of reactions with bacteriophages. The five types and their strains were qualitatively similar to one another in morphology, cultural characteristics, and diagnostic biochemical reactions and to a named culture of *P. aeruginosa* from the American Type Culture Collection, and their characteristics agreed with those listed by most authors in the literature as diagnostic of the species. The strains and types showed some quantitative differences in production of pyocyanin, development of iridescence, and degree of haemolysis of blood and of hydrolysis of casein. The symptoms of infection and the loss from mortality are described. The factors influencing the spread of the infection are discussed and methods of suppression of the disease are recommended. The disease originates from a small number of bacteria intimately associated with a very low percentage of grasshopper eggs collected in the field. Nymphs emerging from these eggs die shortly after emergence and contaminate the food supply. Grasshoppers are normally infected by ingesting food contaminated by the bacteria but the bacteria do not multiply in the gut and most of them are rapidly killed or eliminated. The bacteria are not actively invasive, but when a small number gain access to the haemocoel they increase in numbers to about 1×10^9 per insect. Shortly thereafter the host dies from the metabolic activities of the parasites, most probably as a result of protein digestion by the strong proteolytic enzymes of the bacterium. There is no evidence that the bacteria produce a specific toxin, and grasshoppers are more resistant to the poisonous action of pyocyanin than are mice.

Introduction

During investigations of the microorganisms associated with grasshoppers, the bacterium *Pseudomonas aeruginosa* (Schroeter) Migula was isolated repeatedly from grasshopper cultures that were suffering a high degree of mortality in the laboratory. Healthy grasshoppers in laboratory-reared or field-collected samples were free of this organism. In an extended bacteriological examination of 72 field collections made during four seasons in the Canadian provinces from Ontario to British Columbia, the bacterium was not isolated from either dead or living grasshoppers.

The literature contains few references to the association of *P. aeruginosa* with insects. Cao (5) and Duncan (8) isolated it from the gut of *Melolontha vulgaris* F. and *Stomoxys calcitrans* (L.) respectively. One of the strains studied by Jordan (10) was isolated from the exudate of a sick caterpillar. Lepesme (14) found it associated with mortality in laboratory cultures of the desert locust, *Schistocera gregaria* Forsk., but did not attribute the mortality to it. Metalnikov (16) and Cameron (4) found the wax moth, *Galleria mellonella* (L.), experimentally susceptible to small intracoelomic doses and Sawamura (19) stated that the silkworm, *Bombyx mori* (L.), was

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susceptible to inoculation. Cao (6), Bacot (1), and Ledingham (12) found that *P. aeruginosa* fed to the larvae of various flies might persist to the adult stage of the insect, but Couvreur and Chahovitch (7) stated that *in vitro* the digestive juices and blood of the silkworm destroyed the organism.

In North America the bacterium has been associated with mortality in most of the laboratories where grasshoppers have been reared for experiment. The disease has occurred in laboratories at Kingston and Belleville, Ontario; Brandon, Manitoba; Saskatoon, Saskatchewan; Lethbridge, Alberta; and Bozeman, Montana. It was found infecting eight cultures of *Melanoplus mexicanus* (Sauss.), six of *M. bivittatus* (Say), five of *Cammula pellucida* (Scudd.), four of *M. packardii* (Scudd.), and one of cutworms; the frequency of isolation was a measure of the popularity of the host in rearing rather than of its susceptibility to the disease. The adults and all nymphal instars of the hosts are susceptible to infection, and death may occur at any stage.

Description of the Bacterium

A description of the organisms found in grasshoppers and identified as *P. aeruginosa* is given below because authors have disagreed on the identifying characters of the species and the genus. Strains isolated from grasshoppers formed a closely knit group with similar reactions and differed from one another only in the degree to which they displayed certain properties. Strains were separable into five phage types on the basis of their possession of or sensitivity to temperate bacteriophages, as defined by Boyd (2). *P. aeruginosa*, 10145 of the American Type Culture Collection, was similar to the grasshopper strains but was insensitive to the temperate phages and did not carry a symbiotic phage, to which grasshopper strains were sensitive.

P. aeruginosa from grasshoppers was a Gram-negative, highly motile, single or paired rod, about 0.7–0.8 $\mu \times$ 1–2 μ, possessing a single polar flagellum and forming neither capsules nor spores.

Colonies on agar were faintly green, roughly circular, low-convex to flat, with thin, spreading, translucent or transparent peripheries and irregular fimbriated or villous margins. The surface was shiny and irregularly undulate or roughened. Size depended on age and surface moisture; colonies reached 4–5 mm. in diameter in 24 hours and thereafter spread slowly until the agar dried. Variation in colony form appeared to be induced by crowding and the composition of the medium, particularly its moisture content. Only two basic colonial forms were recognized: the spreading form described above, and a non-spreading, small, circular, convex, smooth, finely granular, entire form. On initial isolation all colonies were of the spreading type. The smooth form appeared to be a rare mutation better adapted to low oxygen tension since it occurred on plates seeded from aging broth cultures or from those resulting from several broth-to-broth transfers, but once isolated it bred true. Mucoid or gelatinous variants were not encountered.

In unshaken broth, there was abundant growth and turbidity with some ring formation, a surface pellicle, and later considerable sediment. The

surface layers of broth were light green and the whole of the broth medium, when shaken vigorously, became green because of oxidation of the leuco-reduced form of pyocyanin (26).

Infected grasshoppers, as well as agar and broth cultures, had a characteristic smell variously described as similar to new-mown hay, grapes, or trimethylamine (24). The smell was strong on newly isolated strains and became milder on old strains that were repeatedly subcultured.

All strains produced the water-soluble pigments fluorescin and pyocyanin which are characteristic of the organism and which color agar media various shades of green. The quality and intensity of the color varied from strain to strain and was determined by the amount of the blue pyocyanin produced in proportion to the yellow fluorescin, as shown by Leonard (13). Color production was influenced by the age of the strain and by the components of the agar medium; all strains tended to lose the blue tint after prolonged storage and subculture and all strains gave the maximum of blue tint when plated on Sabouraud maltose agar (Difco) or on the medium of King *et al.* (11). Bacto-nutrient agar (Difco) was unsuitable for color production but nutrient agar compounded of 3 g. of Difco beef extract, 5 g. of Difco Bacto peptone, and 15 g. of Bacto agar per liter at pH 7.4 allowed a good range of color to develop and this formula was used for routine plating, or without agar as a fluid medium. In broth the deepest color (i.e., the greatest production of pyocyanin) occurred in Difco nutrient broth to which had been added 75 g. of cooked potato and 10 g. of glycerol per liter. The best yields of pyocyanin were obtained by growing the bacteria in still culture at 37° C. in 500 ml. flasks containing 100 ml. of potato–glycerol nutrient broth and harvesting the growth after 10 days, at which time the broth turned deep green when shaken. No color developed if the flasks were continuously shaken by machine during incubation, presumably because the pyocyanin was oxidized to a colorless compound (13). The pyocyanin was extracted with chloroform (25), converted to the acid salt by shaking with *N*/10 HCl, purified by interconversion from the water-soluble to the chloroform-soluble state, and crystallized after neutralization of the third water solution. Pyocyanin crystallized out as long, dark blue needles. Some strains produced yields of 2.8 mg. of pyocyanin per 100 ml. of culture.

Pyorubin, a red pigment produced by some strains of *P. aeruginosa*, according to Leonard (13) and Meader *et al.* (15), was not produced by the grasshopper strains. After the pyocyanin was extracted from broth cultures with chloroform, the residue retained a small amount of the yellow pigment (fluorescin) without traces of red. On agar media, after prolonged incubation, the color sometimes changed from blue-green to purple; this suggested the presence of small quantities of red pigment, but this was not investigated. Turfitt (22) claimed that pyorubin was not a distinct pigment but fluorescent pigment in high concentration mixed with partially oxidized pyocyanin.

The ability of strains of *P. aeruginosa* to produce acid from carbohydrates in the medium was tested on 1% carbohydrate broth incorporating bromcresol

purple as the indicator according to the method recommended in *Manual of Methods for Pure Culture Study of Bacteria* (21). Within 7 days all strains produced acid without gas from glucose, galactose, mannose, and xylose, and some strains fermented *l*-arabinose (P11-1, 249-1-A, 251-4-3). Strains varied somewhat in the speed of acid production. None of the strains produced acid from cellobiose, fructose, lactose, maltose, raffinose, rhamnose, sucrose, trehalose, adonitol, dulcitol, glycerol, inositol, mannitol, sorbitol, dextrin, inulin, salicin, or starch. These results agree with those of Ringen and Drake (17), who used agar slants, but most authors reported that the bacterium produced acid from glucose only. The failure of most workers to demonstrate acid production from other sugars was shown by Salvin and Lewis (18) to be a result of neutralization of the acid by the ammonia released in proteinaceous media and they demonstrated the production of acid from glucose, galactose, mannose, xylose, arabinose, and glycerol in a medium devoid of organic nitrogen. Therefore, the strains from grasshoppers were peculiar because they produced acid from most of these substrates despite the organic nitrogen content of the medium.

Other biochemical tests were carried out according to procedures outlined in *Manual of Methods for Pure Culture Study of Bacteria* (21). The grasshopper strains behaved uniformly. Acetylmethylcarbinol was not produced; the methyl red test was negative; indole was not produced from tryptophan; hydrogen sulphide was not produced; urease was absent; milk was peptonized with an alkaline reaction; gelatin was liquefied; methylene blue was reduced; ammonia was produced; nitrates were reduced to nitrites and the nitrites broken down to nitrogen; and strains grew in media containing citrate as the sole source of carbon. These reactions agree with those reported for *P. aeruginosa* by several authors (3, 18, 24).

All strains grew at a temperature of 42° C. but not at 5° C. and growth was optimum in the range 30–37° C. All strains grew aerobically. None grew under anaerobic conditions in Brewer anaerobic jars in an atmosphere of hydrogen or on Brewer anaerobic agar in Brewer plates. However, growth occurred under low oxygen tensions in deep broth cultures and in Robertson's meat medium.

All strains grew in media with an initial pH range of 5.5 to 8.0 but the initiation of growth was favored by a pH of 7.2 to 7.4. All nutrient broth cultures, regardless of initial pH, reached a pH of approximately 8.5 to 8.6 after 24 to 48 hours' growth at 37° C.

All strains were killed by exposure to a temperature of 56° C. for 0.5 hours. *P. aeruginosa* was very sensitive to desiccation; only 10% of the bacteria in broth culture survived drying at room temperature and 30–40% relative humidity for 24 hours. However, the bacteria survived in concentrated suspension in distilled water for long periods. This sensitivity to drying led to a search for a protective agent that would increase its tolerance to desiccation.

Grasshopper strains were pathogenic for laboratory animals. Intraperitoneal injection of 1 ml. of culture containing 2.8×10^7 bacteria killed 20-g. mice in 24–48 hours.

The bacteria were readily grown for experimental use in nutrient broth. Cultures grown in 5 ml. of broth in tubes 15 mm. in diameter produced counts of $1.0–3.0 \times 10^9$ bacteria per ml. in 18–24 hours. Higher populations occurred in 500 ml. flasks containing 100 ml. of broth; after 18–24 hours, counts ranged from 7.0 to 9.0×10^9 per ml. and a maximum population of $1.0–3.0 \times 10^{10}$ bacteria per ml. was reached in 25–30 hours. Higher populations were not produced by continuous aeration by shaking during incubation.

Differences between Strains

The major difference between the grasshopper strains of *P. aeruginosa* was their reaction to temperate bacteriophages. This allowed them to be grouped into five phage types. They differed quantitatively in color on agar media, in amount of pyocyanin produced, in degree of haemolysis of blood and of peptonization of milk, and in presence or absence of iridescence. Most of the confusion in the literature regarding classification centers around the acceptance of pyocyanin production as a requisite for inclusion in the species and the difficulty of classifying strains in which pyocyanin production is minimal or lacking. As a preliminary to studies of comparative virulence, the phage types were compared for color, pyocyanin production, haemolysis, and peptonization of milk with *P. aeruginosa* A.T.C.C. 10145 and with several strains of related bacteria from grasshoppers. The strains are compared in Table I.

The development of water-soluble pigment was observed on nutrient agar, Sabouraud maltose agar, blood agar, and milk agar. Pyocyanin production was measured by growing the bacteria in potato–glycerol broth, extracting the pyocyanin, and estimating the amount colorimetrically in arbitrary units. The haemolytic action was estimated by the width of the zone of beta-haemolysis surrounding the growth on rabbit blood agar plates after 48 hours, and the peptonization of milk (casein hydrolysis) was estimated by the width of the clear zone surrounding the growth on milk agar plates after 48 hours. The presence of iridescence was observed on thick nutrient agar plates after 48 hours; the iridescent plaques appeared as lysed areas of growth covered by a metallic sheen (23).

Table I shows that all strains were blue-green on Sabouraud maltose agar but that the iridescent strains produced the greatest amount of pyocyanin as indicated by their blue-green color on other agar media and by their pyocyanin production in broth. Culture A.T.C.C. 10145 was an exception; although iridescent and producing much blue-green pigment on agar media, it gave a low yield of extractable pyocyanin from broth culture. Warner (23) stated that iridescent strains usually produced more pigment. There was no evident relationship between the degree of haemolysis or casein hydrolysis and any of the other variable factors. Strains of the same phage type might

TABLE I

COMPARISON OF STRAINS OF *Pseudomonas* SPP. ON THE BASIS OF COLOR, IRIDESCENCE, PYOCYANIN PRODUCTION, HAEMOLYSIS, AND CASEIN HYDROLYSIS

Strain	Phage type	Color on agar*				Iridescence†	Pyocyanin production in broth, units	Haemolysis of blood agar, zone width, mm.	Hydrolysis of casein agar, zone width, mm.
		Nutrient	Blood	Milk	Sab.				
P. aeruginosa									
330–10–A	284–6–A	BG	BG	BG	BG	I	12.6	3	7
251–4–3	251–4–3	BG	BG	BG	BG	I	12.2	3	6
1–1–A	1–1–A	BG	BG	BG	BG	I	11.6	2	4
46–1–A	46–4–B	BG	BG	BG	BG	I	10.5	2	6
249–1–A	249–1–A	G	G	YG	BG	—	9.8	4	6
284–6–A	284–6–A	G	G	YG	BG	—	8.2	4	8
284–1–A	284–6–A	G	G	YG	BG	—	3.3	4	9
P–11–1	1–1–A	YG	YG	YG	BG	—	2.3	2	5
207–6–A	251–4–3	G	G	YG	BG	—	1.6	4	9
1–N–12	1–1–A	YG	YG	YG	BG	—	1.0	2	2
A.T.C.C.–10145	10145	BG	G	BG	BG	I	1.0	1	4
P. fluorescens									
249–4–C		Faintly YG	W	Faintly YG	W		0	0	2
Pseudomonas sp.									
43–3–D		Faintly YG	W	W	NG		0	4	0
51–2–A		Faintly YG	W	W	W		0	0	0

*BG, blue-green; YG, yellow-green; G, green; W, colorless; NG, no growth.
†I, iridescent.

vary widely in the degree to which they displayed other characteristics. Strain P11-1 was derived from 1-1-A by 11 successive grasshopper passages, and lost much of its color in the process. Strain 1-N-12 was a smooth colony form derived from 1-1-A after selection from old broth cultures; it was typical of smooth strains in having little color, producing little pyocyanin, and having reduced powers of haemolysis or hydrolysis. All strains identified as *P. aeruginosa* produced some extractable pyocyanin in broth culture.

Strain 249-4-C was identified as *P. fluorescens* Migula; it did not produce pyocyanin on Sabouraud maltose agar or in potato–glycerol broth but produced a faint yellow to yellow-green color on nutrient agar. Though similar to *P. aeruginosa* in most reactions, it did not haemolyze blood, liquefy gelatin, produce acid from galactose, or smell of trimethylamine. It differed from typical strains of *P. aeruginosa* by growing at 5° C. but not at 42 °C. and was insensitive to the phages. Wilson and Miles (24) separate *P. fluorescens* from *P. aeruginosa* by its failure to grow at 42° C. and its growth at 5° C., by lack of pyocyanin production, and by usual failure to liquefy gelatin.

Culture 43-3-D was typical of several isolations suspected of being *P. aeruginosa* that had lost its ability to produce color. Colonies were small, convex, with thin spreading edges and granular, ground-glass surfaces, and cultures produced a strong characteristic fruity odor described as similar to pineapple or ripe apples. On initial isolation they produced a very faint greening of agar media and the strains resembled *P. aeruginosa* in morphology and biochemical reactions, but they lost most of their activity on storage and subculture while retaining their haemolytic properties; they did not grow at either 5° C. or 42° C. and failed to grow on Sabouraud maltose agar; they were insensitive to *Pseudomonas* phages and were mildly pathogenic when inoculated into grasshoppers. They were tentatively classified as *Pseudomonas* sp.

Culture 51-2-A was typical of several isolations that sometimes produced a faint yellow-green color on agar media and that were similar to *P. aeruginosa* morphologically and in many biochemical characters. Colonies were smooth, non-spreading, and flat with internal greenish reflecting planes and lacked odor. Cultures differed from *P. aeruginosa* in failing to liquefy gelatin, peptonize milk, reduce nitrates, or produce haemolysis. Cultures were insensitive to *Pseudomonas* phages and non-pathogenic to grasshoppers on inoculation. They were tentatively classified as *Pseudomonas* sp. Thus none of the cultures isolated from grasshoppers may be considered as strains of *P. aeruginosa* that had lost all ability to produce pyocyanin.

The Disease in Laboratory Cultures

The first noticeable sign of the disease in the laboratory is an abnormally high mortality of the grasshoppers. Normally this occurs first among first and second instar nymphs but, where cultures of mixed age are established, death of the young nymphs is often unnoticed and the presence of disease is indicated by the dead bodies of older nymphs and adults.

Diseased grasshoppers show no symptoms of infection until shortly before death, when they become sluggish. They fall to the bottom of the cage, where they lie on their sides and make slow movements of the legs until they die. At death the body is flaccid and the tissues soft and wet, showing that considerable decomposition has already occurred; body fluid at this time contains huge numbers of bacteria. The bacteria continue to multiply rapidly after death and the body contents are converted into a viscous liquid in which traces of the organs can be recognized with difficulty, if at all. The grasshoppers have the characteristic odor of *P. aeruginosa*. Typically, dead insects have a rosy-pink color and, on dissection, the remnants of the Malpighian tubules are outlined in deep red. However, color did not always develop or was masked by a deeply pigmented integument, and, moreover, a rosy coloration sometimes occurred with other bacterial and fungous infections. Thus a rosy coloration of dead insects is indicative rather than diagnostic of *P. aeruginosa* infection. The proteolytic action of the bacteria on the grasshopper tissues results in an alkaline pH and the red color is apparently a result of the reaction of the alkali with pigment precursors in the hoppers rather than a result of the production of red pigment by the bacteria. Pyocyanin itself is blue-green under alkaline conditions.

Though the disease in cultures of grasshoppers with a high mortality could usually be diagnosed by the color and odor of the dead insects, the cause of death in individuals was determined by triturating the insect in water and plating the diluted suspension on nutrient agar. The characteristic growth on agar media was recognized as *P. aeruginosa* without further tests. If the insect had died with a typical infection, *P. aeruginosa* occurred on the agar plate in pure or very nearly pure culture. On the other hand, healthy grasshoppers and those that had died from causes such as old age or poor molting produced a mixture of bacterial colonies in which *P. aeruginosa* did not occur. Heavy growth of *P. aeruginosa* in the body cavity of the grasshopper suppressed the multiplication of other bacteria.

The disease spreads among grasshoppers in a single cage by contamination of the food with bacteria exuding from decomposing insects. Dead, infected grasshoppers decompose very quickly on wet surfaces and, when the rearing technique employs dry food and a source of drinking water, an infected hopper that dies on the drinking surface quickly infects the whole lot. Caged grasshoppers feed on their fellows that have died or that are less active, as during molting or in the final stages of disease. These habits are important in spreading the disease. Faecal contamination is not important in spreading the infection because *P. aeruginosa* does not persist in large numbers in the gut, as shown below, and the faeces of individuals with rampant infections are often free of the bacterium.

The infection spreads from one cage to another by transferring apparently healthy but actually infected hoppers and by the use of contaminated food and equipment. There is no evidence that the disease is airborne. Agar plates exposed to the air in proximity to cages of infected grasshoppers

remained sterile for *P. aeruginosa*. Although various bacteriological techniques produce aerosols containing bacteria, *P. aeruginosa* has a low survival rate under these conditions and distribution of the organism on dust particles or in aerosols is limited.

Mortality in infected cultures depended to a large extent on the rearing technique. Sometimes whole cultures suffered nearly 100% mortality and sometimes the disease was confined to one or a few cages. First and second instar nymphs frequently suffered such high mortality that mature nymphs or adults were reared only with difficulty. Infection in older nymphs and adults frequently caused relatively less mortality.

If recognized early, the disease can be suppressed by destroying infected lots and by reducing cannibalism among the grasshoppers. As the disease first attacks young nymphs, segregating these in small lots for several days allows early diagnosis; infected lots may be destroyed and healthy ones combined to form a healthy population of uniform age. Cannibalism can be reduced by providing excess food and room, by grouping together insects of similar age, and by frequent removal of dead or sluggish hoppers. Lettuce or other green food is less readily contaminated than water dishes and thus is a superior source of food moisture. A low humidity (about 30%) in the rearing room and aseptic techniques reduce the spread of infection.

The origin of the disease in laboratory cultures remained obscure until strains of the organism were identified by their reactions to bacteriophages. Though the disease may be transmitted by contaminated equipment and thus persist for long periods in one culture of grasshoppers after another, this did not explain the occurrence of spontaneous infection after freedom from disease for a year or more. At Kingston, where cultures of *P. aeruginosa* were maintained and experiments on artificial infection performed, it was assumed that spontaneous infection occurred by contamination of the food through faulty techniques, but phage typing showed that the strains responsible for spontaneous infection were new strains different from those maintained in culture or used experimentally. A search for the outside source of infection included the bacteriological examination of the ingredients of the dry food and the laboratory water supply, with negative results. As *P. aeruginosa* and some related forms have been isolated from plants, including lettuce (9), the lettuce used as green food was periodically subjected to extensive examination for *P. aeruginosa*. The bacterium was not isolated from lettuce, grain sprouts, dandelion, or other greens used as food. Ringen and Drake (17) found that 11% of human individuals were carriers, and suggested that the normal habitat was the intestinal tract of man, but we failed to implicate the laboratory personnel. Extensive bacteriological examination of grasshopper eggs, egg pods, and soil collected in the field for use in rearing showed that a small number of the bacteria were intimately associated with the eggs of some collections. The bacteria isolated from eggs from one locality differed in phage type from those from other localities and the strains isolated during one year differed from those isolated during other seasons. When

spontaneous infection occurred, the causal bacteria belonged to the same phage type as long as the eggs used to rear the grasshoppers came from the same collection and the same strain of *P. aeruginosa* that caused the disease could be isolated with difficulty and in small numbers from the eggs. Thus *P. aeruginosa* exists in small numbers intimately associated with 1% or less of the grasshopper eggs of some collections. It is not known whether the bacteria occur on or within the egg, or both. The soil itself appears to be free of infection. During incubation of the eggs in the laboratory, a small number of emerging nymphs are infected and die shortly after emergence. When death occurs in nymphal rearing cages, the dead hoppers serve as sources of infection for the whole cage. Invariably some die while still in the incubation dishes, thereby infecting the soil and exposing newly emerging, healthy nymphs to infection. Thus, nymphs emerging towards the end of the incubation period may be heavily infected and cultures established from these nymphs may suffer high or total mortality.

Experimental Infection

Grasshoppers were experimentally infected with *P. aeruginosa* by direct inoculation into the haemocoel and by feeding with contaminated food. By either method variation of the dose produced variation in the percentage infection or mortality, so that dosage–mortality curves were plotted and estimates made of the median lethal dose (LD_{50}) by probit analysis. Most experimental tests were made on cultures of *M. bivittatus* or *C. pellucida*.

By Inoculation into the Haemocoel

Adult grasshoppers were highly susceptible to *P. aeruginosa* inoculated directly into the haemocoel. Usually the LD_{50} was from 10 to 20 bacteria per insect. Grasshoppers, therefore, displayed little resistance to the multiplication and lethal effects of *P. aeruginosa* after invasion of the haemocoel. On the other hand, grasshoppers tolerated intracoelomic inoculation of most of the bacterial flora of their intestinal tracts, and did not develop infection from doses of 10,000 to 100,000 of these bacteria.

Grasshoppers showed considerable individual variation to infection with *P. aeruginosa*. This was indicated by the low slope of dosage–mortality curves. Thus it required a dose 13 to 20 times the LD_{50} to kill 100% of the population.

After the haemocoel was infected, *P. aeruginosa* multiplied until the host was killed by the disease. Multiplication of *P. aeruginosa* in grasshoppers was followed by inoculating one series of grasshoppers with 87 bacteria each and another series with 870. At periodic intervals 5 to 10 hoppers were withdrawn and individual counts made of the number of *P. aeruginosa* per hopper. Fig. 1 shows that the multiplication of *P. aeruginosa* in grasshoppers was similar to that in broth in that there was first a drop in the number of bacteria, then a lag period followed by a period of logarithmic multiplication, and finally a decrease in the growth rate, as the number that can be tolerated

by living grasshoppers was approached. The difference in slope between the curves in grasshoppers and in broth was due to the difference in incubation temperatures, which were 37° C. for broth and 30° C. for the insects. During the logarithmic growth phase in grasshoppers, *P. aeruginosa* divided once every 79 minutes. After 48 hours, living grasshoppers contained about 2.4×10^9 bacteria, but dead grasshoppers contained about 10 times as many. Some deaths occurred 42 hours after infection but most occurred shortly after 48 hours. Grasshoppers remained alive and active while supporting a population of about 10^9 *P. aeruginosa*, but died shortly after this concentration was reached. Immediately after death further rapid multiplication of the bacteria occurred. After the first 12 hours, growth of the bacteria in the body cavity of the grasshopper was independent of the size of the infective dose. During the test, grasshoppers carried in the gut a normal bacterial flora of approximately 5×10^6 bacteria. Growth of *Pseudomonas* in the haemocoel did not suppress the other bacteria until just before the death of the grasshoppers.

Differences in individual resistance to infection of the haemocoel by *P. aeruginosa* and in temperature were the principal factors that determined

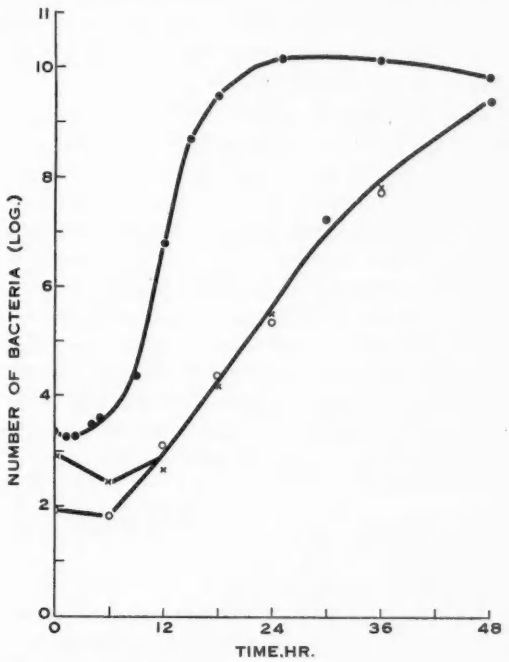

FIG. 1. Growth curves of *P. aeruginosa* in broth and in the haemocoel of grasshoppers: ●, in nutrient broth; ○, in the haemocoel after inoculation with 87 bacteria per hopper; ×, in the haemocoel after inoculation with 870 bacteria per hopper.

the time of death, provided the infective dose was greater than the LD_{50}; at 30° C. from one-third to two-thirds of an infected culture died within 42 to 48 hours after infection and the remainder during the third day. But when the dose was close to or less than the LD_{50}, some grasshoppers survived for 4 to 6 days. In these cases the lag phase of the growth curve was prolonged and the bacteria prevented from actively multiplying, presumably by some resistant action of the host. In some individuals, the bacteria retained their viability and later multiplied logarithmically; in others, the bacteria died and the hosts recovered.

By Feeding

Adult grasshoppers were much more resistant to *P. aeruginosa* when the bacteria were fed than when they were inoculated into the haemocoel. Moreover, the LD_{50}'s varied much more widely for feeding than for inoculation and usually ranged from 8000 to 29,000 bacteria per insect. Nymphs were relatively more susceptible to infection. Most of the bacteria that commonly inhabit the gut of the grasshopper were harmless when fed in massive doses.

Grasshoppers showed even more individual variation to infection with *P. aeruginosa* by feeding than by inoculation. This was indicated by the low slope of the dosage–mortality curves and by the extreme variation in the time of death. Thus it required a dose 10 to 100 times the LD_{50} to kill 100% of the population.

The time of death was subject to greater variation following feeding than following inoculation. When grasshoppers were fed on doses greater than the LD_{50}, most of the deaths occurred within 7 to 21 days. There was poor correlation between the size of the dose and the time of death.

As preliminary work had indicated that *P. aeruginosa* rapidly disappeared from the gut of grasshoppers that had eaten it, the rate of disappearance from the gut was investigated. A number of adult grasshoppers were fed 2.1×10^6 bacteria per hopper and at intervals the guts of 10 hoppers were removed aseptically and the number of *P. aeruginosa* within each determined. Fig. 2 shows an immediate logarithmic decline in numbers for about the first 8 hours after ingestion, followed by lower rates of decline with increased time. *P. aeruginosa* could not be isolated from the gut by routine plating methods 72 hours after ingestion, indicating that, if present, it occurred in very small numbers.

The long period between ingestion of *P. aeruginosa* and death from the disease, the great variation in the length of this latent period between grasshoppers, and the lack of multiplication of the bacterium in the gut and its disappearance from the gut during the latent period suggest that the bacteria exist in very small numbers in the gut in an inactive state, probably in some "pocket" where the diluting action of feeding and elimination is minimal. Under stimuli, as yet poorly understood, the organisms gain access to the haemocoel and cause a fatal infection.

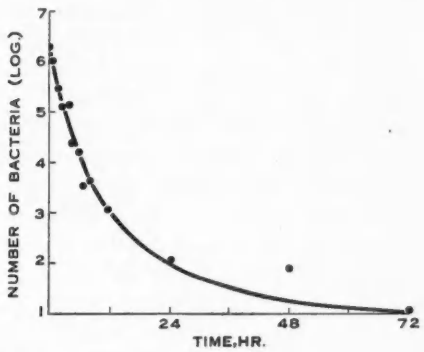

FIG. 2. Survival of *P. aeruginosa* in the gut of grasshoppers.

Preliminary studies of the blood of grasshoppers fed *P. aeruginosa* indicated that the blood remained sterile until 2 or 3 days before death from the infection. After the bacteria first appear in the blood, they multiply rapidly, in a manner similar to that observed in inoculated insects, until a population of about 10^9 bacteria per insect is reached, after which the insects die.

Discussion

Pseudomonas aeruginosa causes a fatal disease in grasshoppers by multiplying to huge numbers in the haemocoel of the host and producing a general infection of the tissues. Although grasshoppers are normally infected by feeding on contaminated food, they show considerable resistance to infection and ingested bacteria are rapidly eliminated from the gut. So long as infection is confined to the gut the host remains healthy, but despite rapid elimination of bacteria from the gut, a small number of bacteria apparently survive for long periods, invade the haemocoel at a later date, and produce a fatal infection. Grasshoppers are susceptible to infection of the haemocoel by very small numbers of *P. aeruginosa*. Once the haemocoel is invaded, the bacteria multiply as in broth culture and the insect dies with general decomposition of the tissues. This pattern of infection does not suggest that *P. aeruginosa* produces a toxin comparable to that of the bacteria causing diphtheria or tetanus; grasshoppers carrying rampant infection of the haemocoel show no symptoms of disease and cannot be distinguished from healthy insects until very shortly before death. On the contrary, the pattern suggests that death of the host is due to the general metabolic activities of the bacteria, their production of enzymes, their utilization of the body substance of the host as food, and their production of waste products. The appearance of the host at death indicates that protein decomposition is the most important of these activities. In this connection it may be noted that *P. aeruginosa* is strongly proteolytic in culture, breaking down gelatin and casein. Although pyocyanin is produced by cultures pathogenic to grasshoppers and is known to be toxic to vertebrates,

there is no evidence that it plays a significant role in the disease of grasshoppers; these insects are resistant to injected doses the equivalent of which kill mice. Grasshoppers showed no ill effects when inoculated with one part of pyocyanin per 10,000 to 100,000 parts of body weight; mice were killed by intraperitoneal doses of the same magnitude. Schoental (20) stated the M.L.D. of pyocyanin for mice was 2 mg.; the average weight of a mouse is about 20 g.

P. aeruginosa demonstrated a low capacity for invading a susceptible tissue, the haemocoel. The highly variable nature of the time–mortality data and the poor correlation between the time of death and the ingested dose argue against a specific mechanism of invasion. Rather, invasion seems to occur very largely by chance. In this connection it is noteworthy that, although grasshopper blood is normally sterile, it is invaded from time to time by various gut bacteria. If *P. aeruginosa* is among these, a fatal infection occurs.

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NUTRITION AND METABOLISM OF MARINE BACTERIA

IV. THE PARTICIPATION OF Na^+, K^+, AND Mg^{++} SALTS IN THE OXIDATION OF EXOGENOUS SUBSTRATES BY A MARINE BACTERIUM[1]

N. TOMLINSON AND ROBERT A. MACLEOD

Abstract

The inorganic ion requirements for the preparation, storage, and oxidative activity toward exogenous substrates of washed cell suspensions of a marine bacterium have been investigated.

Washing with distilled water greatly reduced the activity of cells toward exogenous substrates. There was found to be a relatively non-specific requirement for the presence of inorganic ions in the wash solution for the preparation of cell suspensions that are metabolically active when fresh.

To maintain metabolic activity of the cells on storage, a specific requirement for Na^+, K^+, and Mg^{++} was demonstrated. In addition, Mg^{++} was active in preventing cytolysis. Mn^{++} could replace Mg^{++} in the latter capacity but not in some function of Mg^{++} connected with oxidative processes.

For oxidation of exogenous substrates, a requirement for Na^+ and K^+ but not Mg^{++} was demonstrated. The optimum Na^+ concentration lay between 0.1 and 0.3 *M* and K^+ between 0.01 and 0.1 *M*.

Introduction

Marine bacteria differ from all land forms investigated in requiring a relatively high concentration of Na^+ in the medium for growth (11, 12). In view of this requirement, an investigation of the effects of sodium chloride and certain other inorganic salts on the oxidation of exogenous substrates by a marine bacterium was undertaken with the object of attempting to learn something of the role of these salts in the metabolism of the organism.

The present paper describes results indicating that Na^+ and K^+ together are essential for the oxidation of exogenous substrates by washed cells of a marine bacterium, and in addition that these two ions, together with Mg^{++}, function in preventing the loss of oxidative ability during aging.

Materials and Methods

The organism studied in this investigation was isolated from clams and has been designated B16 in this and previous communications. A marine form according to the definition of Zobell and Upham (18), it has now been tentatively identified as a species of either *Pseudomonas* or *Spirillum*.[2] The method used to carry cultures of the organism and some of its requirements for growth have been described (13).

[1]Manuscript received January 21, 1957.
Contribution from the Fisheries Research Board of Canada, Technological Station, Vancouver, B.C.

[2]We are indebted to Dr. Einar Leifson, Loyola University, Chicago, Ill., for investigating the classification of this organism.

A non-synthetic medium was used. It consisted of artificial sea water[3] supplemented with 0.021% K_2HPO_4–KH_2PO_4 (pH 6.7), 0.1% $(NH_4)_2SO_4$, 0.8% nutrient broth, and 0.5% yeast extract. The medium was sterilized by autoclaving at 15 lb. pressure for 15 minutes.

For the preparation of washed cell suspensions, cultures were grown in 250 ml. portions of medium dispensed in 2-liter Erlenmeyer flasks. Inoculated flasks were incubated at 25° C. on a rotary shaker for 18 hours. Cells were harvested by centrifuging and were washed by decanting the supernatant liquid, resuspending the cells in a volume of washing liquid (*vide infra*) equivalent to one-half the volume of culture from which the cells had been separated, centrifuging the suspension, and again decanting the supernatant liquid. This procedure was repeated twice. The cells were then suspended in a suitable solution and the volume was adjusted to contain a known dry weight of cells. The dry weight was measured by determining the optical density of suspensions with an Evelyn colorimeter (660 mμ filter) then converting the optical density to dry weight by means of a previously calibrated curve.

Conventional techniques (16) were used in Warburg respirometer experiments. The experimental conditions are described in the tables and figures.

Results

Preliminary Investigation of the Influence of Various Washing Solutions and Suspending Media on the Oxidative Activity of Washed Cells

The effects of several washing and suspending media on the oxidative activity of whole cells of strain B16 were determined. The media used were distilled water, 0.0475 *M* sodium phosphate buffer (pH 7.1), 0.4 *M* NaCl (the concentration of this salt in artificial sea water), and artificial sea water (footnote 3). L-Alanine and sodium succinate were selected as substrates because they had been found to be good sources of carbon for growth of the organism, and therefore might be actively oxidized by the cells.

Cells were examined for ability to oxidize the two substrates immediately after the suspensions were prepared, and again after they had been stored for 7, 14, and 22 days at 1° C.

Freshly prepared cells, with the exception of those washed in distilled water, were all most active toward the substrates when the suspending medium contained the salts of artificial sea water (Table I). With cells washed in distilled water, sodium phosphate buffer was the best suspending medium. At no time did water washed cells exhibit oxidative activity with the substrates comparable to the best shown by cells washed in any one of the three solutions. The relatively low activity of these water washed cells toward substrates decreased very rapidly with time at 25° C. It should be noted that large losses of cells occurred during washing with distilled water, apparently due to cytolysis, but the cells remaining at the end of the procedure

[3]Composition of artificial sea water from Lyman and Fleming (9) with slight modifications: NaCl, 0.4 *M*; Na_2SO_4, 0.0275 *M*; $NaHCO_3$, 0.0023 *M*; KCl, 0.0089 *M*; KBr, 0.00081 *M*; $MgCl_2$, 0.052 *M*; $SrCl_2$, 0.00015 *M*; H_3BO_3, 0.00042 *M*.

TABLE I

THE INFLUENCE OF THE COMPOSITION OF THE WASHING SOLUTION AND SUSPENDING MEDIUM ON OXYGEN UPTAKE BY WHOLE CELLS OF B16

Wash solution*	Suspending medium*	Substrate		
		None	Na succinate	L-Alanine
		O_2 uptake in 30 min. (μl.)		
Distilled water	Distilled water	0	32	0
	Buffer	95	142	151
	NaCl	2	19	10
	Sea water	22	36	30
Buffer	Buffer	74	123	146
	NaCl	64	84	68
	Sea Water	92	211	223
NaCl	NaCl	75	68	151
	Sea water	83	264	223
Sea water	Sea water	91	261	228

*Composition of wash solutions and suspending media:
buffer, Na_2HPO_4-NaH_2PO_4, 0.0475 *M*, pH 7.1;
NaCl, 0.4 *M*;
sea water (artificial), see Footnote 3.
Each Warburg vessel contained 7 mg. dry weight of cells in a final volume of 2.8 ml. of suspending medium; 5 μM. of substrate added from sidearm after 15 min. equilibration in water bath; temp. 25° C.; gas phase air; 0.2 ml. 20% KOH in center well.

appeared whole on microscopic examination. However, the effect of sea water on oxygen uptake by these cells, when compared with that of sea water on cells washed in any of the three solutions, strongly suggests that they have suffered some damage during washing in water.

The behavior of the different cell preparations toward the two substrates was very much the same in all cases except that some impairment of the oxidation system for succinate occurred during washing of the cells in dilute buffer.

Very marked differences in activity were found between suspensions in the different media following storage at 1° C. for varying periods of time. Those stored in distilled water lost nearly all their activity toward the two substrates in 7 days. The cells in sodium phosphate buffer lost activity less rapidly, but were nearly inactive at the end of 22 days. Stored in 0.4 *M* NaCl, cells began to show a decrease in activity at 7 days and had lost about 75% of their activity at the 22nd day. In artificial sea water, cells maintained full activity toward L-alanine for 22 days, but lost about 60% of their activity with sodium succinate between 14 and 22 days. Subsequently it was found that the stability of different suspensions in sea water varied, but was always greater than that of suspensions in other solutions.

The Components of Artificial Sea Water Responsible for Its Influence on the Oxidative Activity of Freshly Washed Whole Cells

Since Na^+, K^+, and Mg^{++} are the only cations in artificial sea water known to be required for growth of B16 (11), the effect of their chloride salts, singly

TABLE II

THE ABILITY OF CERTAIN SALT SOLUTIONS TO REPLACE SEA WATER IN INCREASING OXYGEN UPTAKE BY WHOLE CELLS OF B16*

	Substrate		
	None	Na succinate	L-Alanine
Suspending medium†	O_2 uptake in 30 min (μl.)		
Buffer	51	65	118
Buffer, NaCl, KCl	72	131	239
Buffer, NaCl, KCl, $MgCl_2$	62	122	223
Buffer, sea water	62	114	220

*Cells washed in Na_2HPO_4–NaH_2PO_4, 0.0475 *M*, pH 7.1.
†Concentrations in Warburg vessels:
buffer, Na_2HPO_4–NaH_2PO_4, 0.0475 *M*, pH 7.1;
NaCl, 0.46 *M*; KCl, 0.01 *M*; $MgCl_2$; 0.05 *M*.
Other experimental conditions described in Table I.

and in combinations in the suspending medium, on the oxygen uptake of sodium phosphate buffer washed cells was investigated.

Freshly washed cells oxidized both substrates as rapidly when suspended in a solution of NaCl and KCl (at the concentration of their cations present in artificial sea water) as when suspended in artificial sea water (Table II). The addition of $MgCl_2$ to the NaCl–KCl solution reduced the endogenous oxygen uptake to the level observed in sea water, without having any detectable effect on oxidation of the exogenous substrates. The three salts in other combinations, or singly, were not effective in increasing oxygen uptake with exogenous substrates.

The Components of Artificial Sea Water Responsible for Its Influence on the Changes in Oxidative Activity of Whole Cells During Aging

From the above results, it was thought that the NaCl–KCl solution might be a suitable substitute for sea water as a washing and storing medium. However, when cells were stored in NaCl–KCl solution, NaCl–KCl–$MgCl_2$ solution, or in sea water at 1° C., it was found that the cells in NaCl–KCl solution lost activity more rapidly than did those in the other two solutions. Thus it appeared that Mg^{++} was concerned in maintaining the oxidative ability of washed cells.

This effect of Mg^{++} was investigated by preparing cells in several different solutions, then observing the effect of holding them at 25° C. The results are presented in Table III. Cells washed and suspended in NaCl–KCl solution lost their ability to oxidize the two substrates in 20 hours, but showed an increase in endogenous oxygen uptake. Cells suspended in sea water showed much smaller losses in activity toward both substrates in the same period of time. It can be seen that the protective action of sea water on storage could be almost duplicated by adding $MgCl_2$ to the mixture of NaCl

TABLE III

THE INFLUENCE OF Mg^{++} ON THE LOSS OF OXIDATIVE ABILITY BY WASHED CELLS OF B16 DURING STORAGE AT 25° C.

Wash solution*	Substrate	Storage time (hr.) 0	20	44	146
		O_2 uptake in 30 min. (μl.)			
NaCl, KCl	None	52	95	64	19
	Na succinate	204	116	68	40
	L-Alanine	220	94	58	24
NaCl, KCl, $MgCl_2$	None	36	71	86	29
	Na succinate	212	193	156	37
	L-Alanine	218	256	200	29
NaCl, KCl, $MgCl_2$, $MgSO_4$	None	36	60	95	25
	Na succinate	237	226	193	44
	L-Alanine	200	253	227	28
Sea water	None	48	92	116	23
	Na succinate	208	242	196	40
	L-Alanine	221	279	249	28

*The solution is that in which the cells were washed and stored. Salt concentrations: NaCl, 0.46 *M*; KCl, 0.01 *M*; $MgCl_2$, 0.052 *M* (0.0245 *M* when $MgSO_4$ present); $MgSO_4$, 0.0275 *M*.

The suspending medium in the Warburg cups contained the same salts at the same concentrations as were present in the cell suspension being examined, plus Na_2HPO_4–NaH_2PO_4 buffer, 0.0475 *M*, pH 7.1.

Other experimental conditions described in Table I.

and KCl. The further addition of $SO_4^{=}$ (as $MgSO_4$) helped retain the capacity of the cells to attack succinate. During the experiment outlined in Table III, changes in the optical density of the cell suspensions were measured. That of the cells in NaCl–KCl solution decreased from 0.58 to 0.129 (at 660 mμ) during the first 20 hours of storage, while the optical density of the other suspensions decreased to only 0.47 in 6 days. Microscopic examination showed that the suspension in the NaCl–KCl solution after 20 hours was a mixture of cell debris and apparently whole cells, whereas cells in the other solutions remained whole during the 6 day period.

It was found that the increase in endogenous activity observed in aged cell suspensions (Table III) could be accounted for by the presence in the suspending medium of soluble products arising from the lysed cells. The supernatant solution obtained when an aged cell suspension was centrifuged contained material capable of being very rapidly oxidized by the remaining aged cells. Further study revealed that the presence of Na^+ and K^+ as well as Mg^{++} was required to maintain the oxidative activity of the whole cells on aging (Table IV). Since the NaCl concentration was much higher than that of the other two salts in the suspending solution, the ability of sucrose to replace it in the solution was tested. Sucrose showed no capacity to replace NaCl in retaining the oxidative activity of the resting cells with age.

TABLE IV

THE INFLUENCE OF NaCl, KCl AND SUCROSE ON THE LOSS OF OXIDATIVE ACTIVITY OF WHOLE WASHED CELLS DURING AGING

	Fresh suspension			Aged suspension†		
	Substrate		Δ	Substrate		Δ
	None	L-Alanine		None	L-Alanine	
Storage solution*	O_2 uptake in 60 min. (μl.)					
$MgCl_2$	78	269	191	18	21	3
$MgCl_2$, NaCl	76	256	180	37	73	36
$MgCl_2$, KCl	86	289	203	26	28	2
$MgCl_2$, KCl, sucrose	70	257	187	15	8	−7
$MgCl_2$, KCl, NaCl	81	283	202	50	148	98

*Concentrations in storage solutions: $MgCl_2$, 0.052 *M*; NaCl, 0.1 *M*; KCl, 0.01 *M*; sucrose, 0.2 *M*.

†Cell suspensions stored 20 hr. at 25° C.

Cells were washed three times in 0.052 *M* $MgCl_2$, then suspended in storage solution indicated. The Warburg vessels received 1 ml. of the suspension being examined (7 mg. dry weight of cells), and additions such that each vessel contained $MgCl_2$, NaCl, and KCl in final concentrations of 0.052 *M*, 0.1 *M*, and 0.01 *M*, respectively. With the suspension stored in the sucrose containing solution, sucrose was present in the flasks in addition at a concentration of 0.2 *M*. Other experimental conditions as in Table I.

Detailed Examination of the Influence of NaCl and KCl on Oxygen Uptake by Whole Cells

To examine in detail the influence of NaCl and KCl on the respiratory activities of B16, advantage was taken of the fact that whole cells capable of excellent oxidative activity when fresh could be obtained by washing the cells in 0.052 *M* $MgCl_2$. These washed suspensions contained 13 to 15 μg. K^+ and less than 2 μg. Na^+, determined by flame spectrophotometry, per ml. of suspension containing 7 mg. dry weight of cells (cf. 2).

The effect of various concentrations of NaCl and KCl, separately and in different combinations, on the oxidation of L-alanine, sodium succinate, and D-glucose was examined. Some of the results obtained with L-alanine as substrate are presented in Figs. 1–3. The data of Fig. 1 demonstrate that with a constant KCl concentration the rate of oxidation of the substrate is controlled by the concentration of NaCl in the medium, and that the rate remains essentially constant for at least 1 hour after the substrate is tipped from the sidearm, unless the substrate is exhausted earlier. The same has been found to be true for variations in the concentration of KCl in the medium when the NaCl concentration is held constant. Thus the data of Figs. 2 and 3 present changes in rate of oxygen uptake occurring in response to different NaCl or KCl concentrations, and not the effect of losses in enzyme resulting from denaturation under adverse conditions.

The response of cells to Na^+ in the presence of varying levels of K^+, using L-alanine as substrate, is shown in Fig. 2. In the presence or absence of added K^+ the rate of oxidation increased to a maximum and then decreased as

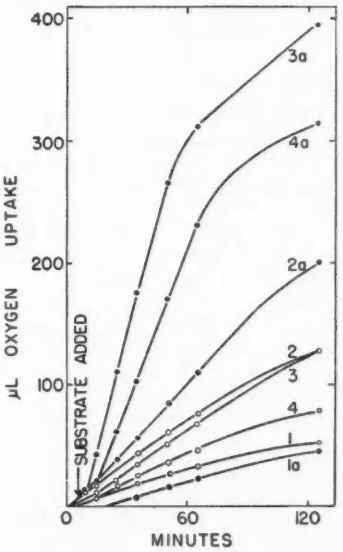

FIG. 1. The influence of NaCl concentration on the endogenous respiration of, and L-alanine oxidation by, marine bacterium B16. O_2 uptake: O———O, endogenous; ●———●, with L-alanine as substrate.

NaCl concentrations: Curves 1 and 1*a*, 0.0;
Curves 2 and 2*a*, 0.03 *M*;
Curves 3 and 3*a*, 0.2 *M*;
Curves 4 and 4*a*, 0.6 *M*.

Each Warburg vessel contained 7 mg. dry weight of cells washed in 0.052 *M* $MgCl_2$, and suspended in a solution containing $MgCl_2$, 0.052 *M*; KCl, 0.01 *M*; and NaCl as indicated above. Other conditions described in Table I.

the Na^+ level was increased. A higher level of Na^+ was required for maximum rate of oxygen uptake in the presence of the substrate than in its absence. As the K^+ level was increased progressively from a suboptimal level (Fig. 2, A to E) the amount of Na^+ required for maximum rate of oxygen uptake in the presence of the substrate was increased slightly (from 0.2 to 0.25 *M*) and the amount of Na^+ required to produce inhibition also increased, indicating some antagonism between Na^+ and K^+. That K^+ may also have capacity to carry out some of the functions of Na^+ is evident from the fact that there was a considerably greater rate of oxygen uptake by cells at suboptimal concentrations of Na^+ (0.03 to 0.1 *M*) with K^+ added at the 0.4 *M* level as compared with that by cells in the same Na^+ concentrations but with levels of K^+ of 0.01 *M* and lower.

The response of the resting cells to K^+ at a constant level of Na^+ in the suspending medium is shown in Fig. 3. In the absence of added K^+ a slow oxidation of substrate was observed which could be accounted for by the small amount of K^+ known to be retained by the cell suspension after washing. The addition of even small amounts of K^+ produced a marked increase in the rate of oxidation of the substrate.

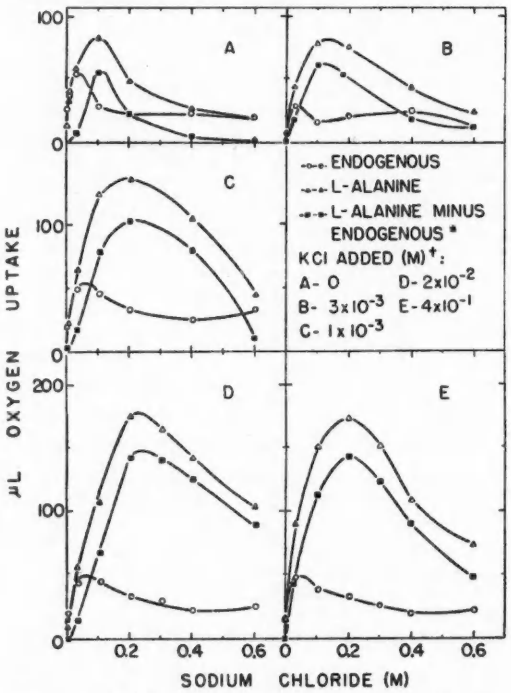

FIG. 2. The influence of various concentrations of NaCl and KCl on the endogenous respiration of, and L-alanine oxidation by, marine bacterium B16. Each Warburg vessel contained 7 mg. dry weight of cells washed and suspended in 0.052 *M* $MgCl_2$ solution, to which was added NaCl and KCl as indicated. Other conditions described in Table I.

*Tomlinson and MacLeod (14).

†The washed cell suspensions used contained sufficient K^+ to bring the concentration of this ion to 1.3×10^{-4} *M* in each vessel before the addition of KCl.

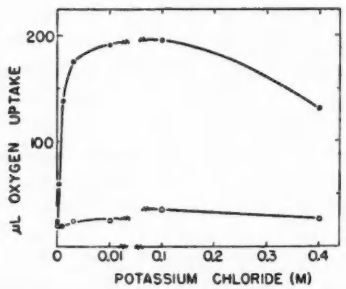

FIG. 3. The influence of various concentrations of KCl on the endogenous respiration of, and L-alanine oxidation by, marine bacterium B16. O_2 uptake: O———O, endogenous; ●———●, with L-alanine as substrate. Concentrations in Warburg vessels: NaCl, 0.02 *M*; $MgCl_2$, 0.052 *M*; KCl added as indicated. Other conditions described in Fig. 2.

Sucrose as a Substitute for NaCl in the Oxidation of Exogenous Substrates by Whole Cells of B16

In view of the report that " . . . moderate dilutions of isotonic sucrose give the same per cent reduction of luminescence and respiration (of a marine bacterium) as moderate dilutions of sea water . . . " (7) and of the recent finding by MacLeod and Onofrey (11) that sucrose exhibits a limited sparing effect on the requirement for Na^+ by B16 for growth, it was decided to investigate the possibility that sucrose might be capable of replacing NaCl, at least partially, in its effect on the oxidation of exogenous substrates by whole cells. B16 grown in the complex medium used in this work does not oxidize sucrose.

TABLE V

SUCROSE AS A SUBSTITUTE FOR NaCl IN THE OXIDATION OF L-ALANINE BY WHOLE CELLS OF B16

Addition to suspending medium*	Substrate		Δ
	None	L-Alanine	
	O_2 uptake in 30 min. (μl.)		
None	56	145	89
NaCl to 0.2 *M*	42	222	180
KCl to 0.11 *M*	43	171	128
Sucrose to 0.1 *M*	45	150	105
" to 0.2 *M*	33	136	103
" to 0.4 *M*	18	41	23

*Each Warburg vessel contained 7 mg. dry weight of cells (washed in $MgCl_2$, 0.052 *M*); $MgCl_2$, 0.052 *M*; NaCl, 0.1 *M*; KCl, 0.01 *M*, together with the additions indicated above. Experimental conditions as described in Table I.

The data of Table V show that under conditions where increasing the concentration of NaCl from 0.1 to 0.2 *M* doubled the rate of oxidation of L-alanine, and where 0.1 *M* KCl had a marked ability to perform the same function, the substitution of 0.1 or 0.2 *M* sucrose for the additional 0.1 *M* NaCl produced only a very small increase in the rate of oxidation of L-alanine. The substitution of 0.4 *M* sucrose was distinctly inhibitory. As 0.2 and 0.4 *M* sucrose are concentrations that exhibited a sparing effect for NaCl in the growth of B16 (11) it appears that this effect cannot be attributed to a favorable influence on the rate of oxidation of exogenous substrates by the cells.

Mn^{++} as a Substitute for Mg^{++} in Preventing Lysis and Loss of Oxidative Activity by Whole Cells of B16

In view of the known ability of Mn^{++} to substitute for Mg^{++} in preventing the autolysis of a terrestrial bacterium (10), it was of interest to see if a similar effect might be observed with a marine form. Cells were washed and suspended as usual in NaCl–KCl, NaCl–KCl–$MgSO_4$, and in NaCl–KCl–$MnSO_4$ solutions. The suspensions were incubated at 25° C. for 20 hours

then examined for changes in optical density and in oxidative ability. After 20 hours' storage the suspension in NaCl–KCl solution was apparently inactive toward the substrates, as usual, and the activity toward the substrates was not increased by the addition of Mg^{++}. The suspension of NaCl–KCl plus $MnSO_4$ took up no oxygen, but when Mg^{++} was added some oxygen uptake occurred and it was evident that the suspension had retained more oxidative activity towards the substrates than had the suspension in NaCl–KCl solution. This activity, however, was much lower than that of cells stored in the solution containing Mg^{++}. Examination of the suspensions showed that Mn^{++} was at least as effective in preventing the lysis of cells as was Mg^{++}. It thus appeared that Mn^{++} could replace Mg^{++} in preventing cell lysis, but could not replace it in some function related to the oxidative activity of the cells.

Discussion

MacLeod and Onofrey (11) have shown that bacteria from marine sources have salt requirements for growth different from those of terrestrial forms. The present findings indicate that other differences also exist between whole cells of bacteria from these sources. These differences are apparent in the inorganic ion composition of the washing solutions necessary for use in the preparation of metabolically active whole cell suspensions, in the composition of the solutions capable of maintaining oxidative activity of the cells on storage, and in the inorganic ion requirements of the cells for the oxidation of exogenous substrates.

With many bacteria from terrestrial sources, e.g. *Micrococcus* (8), *Lactobacillus* (15), distilled water has been used as the washing solution to prepare suspensions that in dilute phosphate buffers exhibited satisfactory metabolic activity with exogenous substrates. The cells of the marine bacterium studied in the present work, however, were found to possess negligible oxidative activity toward exogenous substrates after washing with distilled water, and the activity was not improved by the addition of artificial sea water to the suspending medium. The results indicate also that any of the salt solutions tested, NaCl, $MgCl_2$, or phosphate buffers, could be used to prepare cell suspensions of B16 metabolically active when fresh. There was thus a definite though apparently non-specific requirement for the presence of ions in the wash solution. Johnson (5) has previously reported that distilled water was unsatisfactory for use in washing marine bacteria.

The ion requirements were highly specific, however, for the maintenance of oxidative activity of washed cells on storage. Na^+, K^+, and Mg^{++} have all been shown to be required by organism B16. Mg^{++}, in addition, played a part in preventing lysis of the cells on storage. The relation, if any, between these two roles of Mg^{++}, however, cannot be established by the results presented here. Goucher *et al.* (3, 4) have shown that the maintenance of the integrity of the respiratory system of *Azotobacter agile* is dependent on the Mg^{++} content of the suspending medium, though no reference was made to the effect of the ion on cellular structure. In any event, although Mg^{++}

does have a similar function in both land and marine forms in maintaining respiratory activity on aging, no evidence of a requirement for Na^+ and K^+ in this capacity in land forms has been reported. Johnson and Harvey (6) have reported that Mg^{++} affected the volume, rate of sedimentation, and optical appearance of cells of *Achromobacter fischeri* in dilute solution.

For the oxidation of exogenous substrates, a requirement for the addition of Na^+ and K^+ but not Mg^{++} to the suspending medium has been demonstrated for the marine bacterium B16. This requirement for Na^+ and K^+ together for the rapid oxidation of exogenous substrates has, to our knowledge, not been observed with a terrestrial bacterium and does not appear to have been recognized in the past with marine bacteria (1, 5) or halotolerant forms (17).

Johnson and Harvey (7), working with a marine bacterium, *A. fischeri*, and Yamada and Shiio (17) with a halotolerant bacterium, *Bacillus pumilus*, concluded that the function of NaCl and KCl in the respiration of these organisms was related to osmotic pressure. In the first instance, the conclusion was based chiefly on the fact that sucrose could largely replace NaCl, KCl, or sea water in their effects on the respiration of the cells. However, in the present work with marine bacterium B16, sucrose failed to substitute for NaCl in the oxidation of exogenous substrates by whole cells and in the preservation of their oxidative activity on aging.

The role of NaCl and KCl in the metabolism of B16 is more complex than that contemplated for these salts in *A. fischeri* and *B. pumilus*. In B16 there is a function specific for K^+ that appears to be inhibited by Na^+, a second function that either ion can perform, and a third function specific for Na^+ for which the apparent optimum concentration of this ion is nearly independent of KCl concentrations between 0.001 and 0.4 *M*. This may, of course, be an oversimplification, as these functions are only the minimum number that can be distinguished in B16 from the results recorded in Fig. 2.

Claren (1) reported a stimulation of the oxidation of glucose and fumarate by a marine bacterium suspended in NaCl solution on the addition of a mixed Na–K phosphate buffer, but attributed the effect to phosphate. Johnson (5) reported a phosphate buffer to be stimulatory to the respiration of marine bacteria suspended in NaCl solution, but the cations of the buffer were not mentioned. Similarly, Yamada and Shiio (17) reported stimulation of respiration of a halotolerant bacterium by either NaCl or KCl in a phosphate buffer of unspecified cation content. Investigation of the effect of phosphate buffers with B16 has shown that for freshly washed cells it is the cations of the buffer that are responsible for its effects and not the phosphate ion. Thus, NaCl stimulated substrate oxidation by cells in potassium phosphate buffer, but not by those in sodium phosphate, while for KCl the reverse was true. In addition, it has been found that for cells freshly washed in NaCl–KCl solution, phosphate buffer can be omitted from the Warburg flask without significant effect on oxidation of L-alanine, sodium succinate, or D-glucose.

These results suggest that the results of Claren (1) were probably due to the K^+ of the buffer, and indicate that a similar explanation may apply to the results of Johnson (5) and Yamada and Shiio (17).

At least two explanations are possible for the fact that the NaCl concentrations necessary for optimum endogenous respiration and for oxidation of exogenous substrates are different (Fig. 2). At first this implies a different optimum NaCl concentration for activity of some enzyme(s) concerned with oxidation as opposed to others concerned, for instance, with activation of exogenous substrates. Alternatively, however, it may be that at low concentrations of NaCl some mechanism associated with the destruction of cellular components may be favored and this could possibly free materials within the cells oxidizable by them, thus bringing about the apparent increase in endogenous respiration.

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SCREENING OF CARBOHYDRATES FOR SPORULATION OF BACILLI IN FLUID MEDIUM[1]

S. K. Majumder and M. C. Padma

Abstract

Carbohydrates that are used in fermentation media were screened for sporogenic activities on *Bacillus cereus*, *B. subtilis*, and *B. megaterium*. Out of the 15 carbohydrates tested, *d*-xylose (0.5%) in nutrient broth exerted maximum sporogenic stimulation on the test organisms. In shake culture experiment with *B. subtilis* the sporogenic action of xylose was evident even during the active multiplication of the organism in the medium.

Introduction

As a prerequisite for carrying out fundamental studies on the chemical composition and biology of endospores, studies have been conducted by various workers (2, 4–7, 11, 16, 17) on the sporulation of bacilli in fluid media. Sporogenesis in a *Bacillus* sp. has been considered by Knaysi (9, 10), Hardwick and Foster (7), and Foster and Perry (5) as a starvation phenomenon. A healthy vegetative cell of a *Bacillus* sp. would undergo the metamorphosis to endospore "in the absence of exogenous nutrition" (7) or "facing starvation" (10).

Reports are available in the literature on the effects of a few carbohydrates on sporulation of bacilli in fluid media (4, 8, 10–12). Foster and Helligman (4) observed a stimulatory effect of glucose in *l*-glutamate salts medium on the sporulation of *Bacillus cereus*. On the other hand Kaplan and Williams (8), who studied the spore formation of *Clostridium sporogenes*, showed that the presence of 1% fermentable sugar in nutrient agar results in almost complete inhibition of sporulation. But no systematic screening of the carbohydrates for sporulation of bacilli appears to have been conducted with a view to using them in a fluid medium. The authors have therefore endeavored to compare the fermentation media used in taxonomic studies on bacteria for their sporogenic properties.

Experimental

For the screening of carbohydrates for sporogenic activity 10 ml. volumes of "fermentation broths" in tubes having inverted Durham's tubes were inoculated with a 4 mm. diameter loopful of 24-hour nutrient broth cultures of *Bacillus subtilis* (NCL), *B. cereus* (NCL), and *B. megaterium* (NCL). There were four replicates for each treatment and they were incubated at 37° C. for 4 days. General growth characteristics were noted after incubation. Sporulation data were obtained from stained preparations with the malachite green method of spore staining (1). Vegetative cells, endospores, and liberated spores were counted in each microscopic field from the smear, and average

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counts of 10 fields were taken from each slide. The pH was determined by "spot plate" method, using the indicators of Clark and Lubs (10). The results are given in Table I.

From the results of the above experiment it was evident that xylose exerted a stimulatory influence on sporulation of the bacilli. Further experiments were therefore carried out to examine its effect in aerated cultures. Fifteen milliliters of *d*-xylose broth and basal nutrient broth in 100-ml. Erlenmeyer flasks were inoculated with 0.5 ml. of a 24-hour nutrient broth culture of *B. subtilis*. The cultures were incubated on a reciprocating shaker having 80 strokes per minute at 30° C. Flasks were removed in triplicate after every 24 hours' incubation for observation. The data are presented in Table II.

In the search for sporogenic carbohydrates for *B. cereus*, *B. subtilis*, and *B. megaterium* the results (Table I) indicated that the sugars in the medium exerted differential actions on the growth and sporogenesis of the selected organisms. Of the sugars tested, *d*-xylose was found to give the best yield of spores.

In shake culture experiments with *B. subtilis* (Table II) it was noted that the proportion of vegetative cells undergoing sporulation was comparatively more in xylose nutrient broth than in the nutrient broth control medium. But the cell population was higher in nutrient broth than in the xylose nutrient broth medium after 24 hours' incubation. However, lysis of the cells became pronounced in the nutrient broth control after 24 hours of incubation and resulted in a lower population count after 48 hours. And under similar conditions of inoculation and incubation, xylose in the nutrient broth stimulated better sporulation even within 24 hours of incubation. In xylose nutrient broth the total population reached the peak after the initiation of sporulation in the bacterium. Therefore, some of the cells were multiplying while others underwent sporulation. On this basis, a direct sporogenic action of *d*-xylose in the organism under the experimental conditions seems to be a reasonable conclusion.

Discussion

In normal complex organic media antisporulating factors and antimetabolites are either present or elaborated by bacilli during growth (3). These are known to exert inhibitory effects on sporulation of bacilli (3, 6, 15). Roberts and Baldwin (15) were able to remove the antisporulating factors from peptone media by the pretreatment of media with activated charcoal. Olsen and Scott (12) observed that the incorporation of soluble starch in the medium led to the absorption of antisporulating factors for non-diastatic sporeformers. Our observation on sporogenic stimulation by *d*-xylose in nutrient broth on some of the mesophilic bacilli indicates a scope for further studies.

The present concept that the sporogenesis of a *Bacillus* is primarily dependent on the "depletion of nutrients" in a medium is not solely reconcilable on the basis of the results obtained in our experiments. Sporulation

TABLE I

No.	Carbohydrates*	*Bacillus megaterium*				*Bacillus subtilis*				*Bacillus cereus*			
		Gas	pH	Pellicle	% spore	Gas	pH	Pellicle	% spore	Gas	pH	Pellicle	% spore
1	NB† (no carbohydrate)	–	7.5	+	0	–	7.8	+	0	–	7.2	+	0
2	Arabinose	–	6.0	–	0	–	6.0	+	1.5	–	6.6	+	5
3	Xylose	–	6.0	+	35.4	–	6.9	+	24.6	–	6.6	+	21
4	Glucose	–	5.0	+	1.25	–	5.8	+	0.3	–	5.5	+	0.1
5	Galactose	–	6.5	+	3.7	–	7.2	+	2.5	–	6.8	+	0.4
6	Mannose	–	6.0	–	0	–	6.8	+	0	–	6.7	+	0
7	Lactose	–	7.0	+	0.3	–	7.5	+	0.2	–	6.7	+	0.1
8	Sucrose	–	5.0	+	2.1	–	6.0	+	0	–	5.5	+	0
9	Maltose	–	5.8	+	0	–	6.6	+	0	–	5.7	+	0
10	Starch	–	5.5	+	0	–	6.1	+	1.1	–	6.5	+	2.5
11	Inulin	–	6.3	+	1.1	–	5.9	+	0.4	–	6.6	+	0
12	Dextrin	–	6.0	+	0.2	–	5.8	+	0.1	–	5.6	+	0
13	Glycerol	–	5.8	+	0	–	6.3	+	0	–	5.2	+	0
14	Mannitol	–	6.5	+	0	–	6.5	+	0	–	6.7	+	0
15	Sorbitol	–	7.0	+	1.5	–	6.7	+	0.2	–	6.5	+	0.1

*Fermentation broths with no bromothymol blue indicator (carbohydrates 0.5%).
†Nutrient broth. Beef extract (Difco) 3 g., peptone (Fine Chemicals of Canada) 5 g., and distilled water 1 liter.
+Denotes presence.
–Denotes absence.

TABLE II

SPORULATION OF *B. subtilis* IN SHAKE CULTURE

Incubation in hours	Nutrient broth				Xylose (0.5%) nutrient broth			
	pH	Veg. cells, ml. × 10^{-9}	Spores, ml. × 10^{-9}	Total cells, ml. × 10^{-9}	pH	Veg. cells, ml. × 10^{-9}	Spores, ml. × 10^{-9}	Total cells, ml. × 10^{-9}
Initial	6.9	0.0147	0.0	0.0147	6.9	0.0147	0.0	0.0147
24 hours	8.0	1.101	0.002	1.103	7.3	0.810	0.123	0.933
48 hours	8.6	0.577	0.070	0.647	7.6	0.811	0.368	1.179

of a *Bacillus* even in distilled water has not been regarded as a starvation phenomenon by Powell and Hunter (13). The sporogenic influences of $Fe^{++(+)}$ and Mn^{++} in an organic fluid medium (2), of *p*-amino-benzoic acid in a synthetic medium (18), of the stimulatory effect of carbon dioxide on sporulation of *Bacillus sphaericus* in a complex medium (14), and also of the removal of antisporulating factors from organic complex media (12, 15) to induce sporulation in them indicate the trend for the revision of our widely prevalent concept of the conditions that induce sporulation in bacilli.

Acknowledgment

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OBSERVATIONS ON MICROBIAL FRUCTOSANS AND FRUCTOSANASES[1]

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Abstract

A previously unreported fructosan of *Acetobacter acetigenum* was characterized as similar to that of *Bacillus subtilis-pumilus*, a 2,6-levan. A fructosan of the fungus *Aspergillus sydowi* was characterized as similar to inulin, a 2,1-fructosan. Fructosanases were produced that are specific for either 2,1- or 2,6-linked fructosans. The identification of the fructosans is based on (*a*) the specificity of the fructosanases, (*b*) the rate of movement of fructosan-hydrolyzate products on chromatograms, and (*c*) the presence of detectable amounts of glucose.

Introduction

During the course of an investigation into the mechanism of cellulose synthesis, a fructosan was found in cultures of *Acetobacter acetigenum* grown on sucrose. Members of the genus *Acetobacter* are known to produce cellulose, dextran (4), and starch-like polysaccharides (14) but no reference has been found indicating that they produce fructosans.

The present work describes (*a*) the production of *Acetobacter* fructosan and of a fungal fructosan and (*b*) the production of enzymes that hydrolyze them. A method of characterizing fructosan linkage type (2,1 or 2,6) has been developed which depends upon fructosanases specific for one or the other linkage. Verification of the linkage type is obtained by chromatogramming the oligofructosides derived from the fructosans.

Materials and Methods

Inulin, a 2,1-fructosan found in certain higher plants, was obtained from the Fisher Scientific Company.

Bacillus subtilis-pumilus fructosan was obtained through the courtesy of Professor M. Johnson, University of Wisconsin (11). While this particular preparation (labelled 1624) has not been characterized, *B. subtilis* levans are known to be 2,6-linked (7,1).

Acetobacter acetigenum fructosan was produced from sucrose. Mats of *A. acetigenum* (QM B 1562, NCIB No. 5346 culture originally received from T. K. Walker) were produced by growing the organism in Petri dishes containing 15 ml. of the following medium (per liter): mannitol, 80 g.; sucrose, 20 g.; peptone (Difco), 20 g.; yeast extract (Difco), 20 g.; citric acid.H_2O, 0.83 g.; K_2HPO_4, 1.65 g. The pH of this medium was about 5.2. Cultures were incubated at 30° C. About 4 days after inoculation, a firm mat had formed

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in each dish. These mats were washed well with cold water. Three to six washed mats were placed in cellophane dialysis tubing (5) with about 100 ml. of the following solution (per liter): sucrose, 50 g.; $MgSO_4.7H_2O$, 0.2 g.; citric acid.H_2O, 0.4 g.; K_2HPO_4, 0.72 g.; merthiolate (sodium ethylmercurithiosalicylate), 0.02 g. The dialysis sacs were incubated at room temperature in shallow pans containing an excess of the sucrose solution. The solution in the pans was changed on the third and fifth days. Under these conditions a 12% fructosan solution was produced within the sacs in 2 weeks.

The crude fructosan solution obtained was dialyzed against cold water for several days, filtered through diatomaceous earth, concentrated in a film evaporator (Rotovap) at 40–50° C., and the concentrate poured into nine volumes of acetone. The precipitated fructosan was taken up in a small amount of water and lyophilized.

Aspergillus sydowi (QM 31*c*) fructosan was produced by incubating spores, obtained on potato-dextrose agar, in dialysis sacs containing (per liter): sucrose, 100 g.; KH_2PO_4, 1.36 g.; merthiolate, 0.1 g.; and NaOH in amount sufficient to bring pH of the medium to 5.4. At the end of 1 week a 1.1% fructosan solution was obtained. The solution was dialyzed and added to four volumes of alcohol; the precipitate was taken up in a minimal amount of water and lyophilized.

All four fructosan samples assayed over 100% fructosan on the basis of fructose formed during acid hydrolysis. Ash and "Folin" protein (10) were less than 1% (except for the *Acetobacter* fructosan, which contained about 5% "Folin" protein).

Additional fungi were tested for ability to produce fructosan by incubating spores in the sucrose, phosphate, and merthiolate solution. Fructosan production was accompanied by a change in the appearance of the solution, which became slightly milky.

To test organisms for ability to produce fructosanase they were grown on the two types of fructosans (inulin of 2,1-linkage and *Acetobacter* fructosan, which is shown below to be of 2,6 linkage). The medium contained per liter: yeast extract (Difco), 0.1 g.; KH_2PO_4, 2.0 g.; $(NH_4)_2SO_4$, 1.5 g.; urea, 0.25 g.; $MgSO_4.7\ H_2O$, 0.3 g.; $CaCl_2$, 0.3 g.; fructosan, 4.0 g.; plus the minor elements: Fe, 1 p.p.m.; Zn, 0.8 p.p.m.; Mn, 0.5 p.p.m.; and Co, 0.5 p.p.m. The pH of the medium was adjusted to 6.0 with NaOH. Twenty-five milliliters of medium was used per 125-ml. Erlenmeyer flask. Incubation, usually on a shaker, was at 29° C. At various times during incubation, cell-free filtrates were tested for their ability to hydrolyze fructosans. The fructosanase preparations referred to in this paper are culture filtrates, free of mycelium, to which merthiolate (0.01%) has been added as a preservative, and whose pH has been adjusted to 5.0 with NaOH or citric acid. These preparations contained no reducing sugar or residual fructosans; they did contain other enzymes, some of which are noted below.

Enzyme activities were determined as follows:

(*a*) β-2,1-Fructosanase. To ½ ml. of 0.3% inulin in *M*/20 citrate buffer (pH, 5.4) was added an equal amount of enzyme solution. The mixture was incubated at 50° C. for 1 hour. The reducing sugar liberated was determined as fructose by the dinitrosalicylic acid (DNS) method (13).

(*b*) β-2,6-Fructosanase. As above, using 0.3% *Acetobacter acetigenum* fructosan as substrate.

(*c*) Invertase. As above, using 0.4% sucrose as substrate.

(*d*) Maltase. To 1 ml. of 0.4% maltose, 1 ml. of enzyme solution was added; the mixture was incubated at 50° C. for 1 hour. One milliliter of notatin (glucose oxidase) was added to 1 ml. of the above mixture and shaken at room temperature for 2 hours. Residual maltose was determined by the DNS method.

(*e*) Cellobiase. As for maltase, using 0.4% cellobiose as a substrate.

Fructosan was determined on samples dried overnight at 70° C. after hydrolysis either (*a*) at 100° C. for 30 minutes with 0.5% oxalic acid or (*b*) at 100° C. for 10–60 minutes with *N*/10 HCl. Reducing sugar was determined by the DNS method from a fructose standard curve.

Intermediate products of hydrolysis were obtained by hydrolyzing fructosans (*a*) in 0.33% oxalic acid at 100° C. for 1–3 minutes and (*b*) in fructosanase solutions at 40° C. for 22 hours.

Paper chromatograms were developed for 16–20 hours using isopropanol: acetic acid: water (67:10:23) as solvent. Fructose and fructose containing polymers were detected by spraying the chromatograms with 0.1% resorcinol in 2 *N* HCl (2); glucose was detected with benzidine (8). The rates of movement of compounds are expressed in comparison with the distance moved by fructose, i.e., $R_f = 1.00$ for fructose.

Results

While studying the growth of *Acetobacter acetigenum* (QM B 1562) on various carbon sources, we found a soluble polysaccharide, appearing as a non-mobile fructose-containing spot on chromatograms, when the organism was grown on sucrose. No such material appeared when it was grown on glucose, fructose, glycerol, mannitol, galactose, sorbitol, arabinose, xylose, or sorbose. It was found that non-dividing cells (resting cells) and cell macerates of *A. acetigenum* also produced this polysaccharide from sucrose. Substantial amounts of this material, subsequently identified as a 2,6-fructosan, were produced. *A. xylinum* (QM 1548) also produces fructosan; an acid producer, it gives lower yields and less pure material than *A. acetigenum*. The mechanism of fructosan formation by *Acetobacter* is probably similar to that found in *Aerobacter levanicum* (6).

Since fructosans are poorly known in fungi (as opposed to bacteria), we decided to repeat the work of Kopeloff *et al.* (9) on the *Aspergillus sydowi* fructosan, and to characterize it. Additional fungi were also tested for fructosan production (methods). Three strains of *Aspergillus sydowi* (QM

31*c*, 4*d*, 1990) and three strains of the closely related *A. versicolor* (QM 1989, 4*g* 432) produced fructosan. The poorest among these (QM 1989) produced hülle cells as does *A. nidulans*. The following fungi failed to produce fructosan under the test conditions: *A. nidulans* (QM 1985), *A. silvaticus* (QM 1912), *A. niger* (QM 877), *A. ustus* (QM 6857), *A. janus* (QM 6847) *A. amstelodami* (QM 7091), *Penicillium funiculosum* (QM 474), *Pestalotiopsis, westerdijkii* (QM 381), *Myrothecium verrucaria* (QM 460), and *Trichoderma viride* (QM 6*a*).

Chromatograms of hydrolyzates of the bacterial fructosans showed that the materials contain only fructose. But for a trace of glucose, *Aspergillus* fructosan and inulin also consist entirely of fructose. The glucose that was detected in hydrolyzates of the fungal polysaccharide, and of inulin, was not visible on benzidine-sprayed chromatograms unless these were viewed under ultraviolet light. Bell and Dedonder (1) have also found glucose in inulin, but could not find glucose chromatographically in the bacterial levans they investigated. However, using the glucose oxidase method, they did find traces of glucose in one bacterial levan. It may be that our bacterial levans also contain glucose at this low concentration.

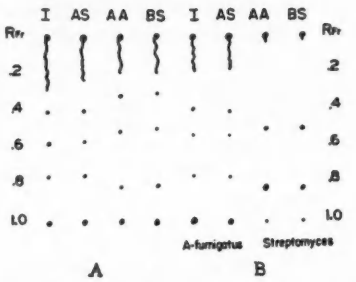

FIG. 1. Hydrolysis products of fructosans; (A) acid hydrolyzates, (B) enzyme hydrolyzates.
I = Inulin (2,1-fructosan)
AS = *Aspergillus sydowi* (QM 31*c*) fructosan
AA = *Acetobacter acetigenum* (QM B 1562) fructosan
BS = *Bacillus subtilis-pumilus* fructosan (2,6-fructosan)

Paper chromatography of the partially hydrolyzed fructosans (Fig. 1) showed that inulin and the *A. sydowi* fructosan are similar and that the two bacterial fructosans are similar. The difructose from the former moves more slowly than the difructose from the bacterial fructosans. From the relationship between log $R_f/(1-R_f)$ and the degree of polymerization (3), it can be shown that acid hydrolysis of inulin and *Aspergillus* fructosan produced fructose, inulobiose, inulotriose, and inulotetrose; and hydrolysis of *Acetobacter* and *Bacillus* fructosans produced fructose, levanbiose, levantriose, and levantetrose. A plot of our data is essentially the same as that of French and Wild (3), even though we used a different solvent system.

To confirm the linkage between the fructose units in *Acetobacter* and in *Aspergillus* fructosans, we attempted to produce enzyme systems that would

specifically hydrolyze either 2,1- or 2,6-linkages; all the fructosans known are of either one or the other type. A total of 50 fungi and bacteria were tested for their ability to utilize fructosans. Like other polysaccharases in fungi, fructosanases are adaptive; that is, little fructosanase (or but a trace) is produced during growth on glucose. Most of the organisms grew well on either inulin or *Acetobacter* fructosan (or both) and so must have the enzymes to utilize them. However, the culture filtrate of most organisms did not contain any detectable fructosanase. Either the fructosanase was not released, or it was rapidly inactivated after release. No fructosanase activity was detected in culture filtrates of the following organisms: *Absidia ramosa* (QM 8*b*); *Aspergillus flavus* (QM 10*e*), *A. luchuensis* series (QM 873), *A. sydowi* (QM 31*c*), *A. unguis* (QM 8*f*); *Basidiomycete* sp. (conidial stage, QM 806); *Botrytis cinerea* (QM 520); *Circinella sydowi* (QM 629); *Chaetomium elatum* (QM 606), *Chaetomium globosum* (QM 459); *Coprinus sclerotigenus* (QM 933); *Monotospora brevis* (QM 1243); *Myrothecium verrucaria* (QM 460); *Paecilomyces varioti* (QM 10*a*); *Penicillium frequentans* (QM 1924), *P. italicum* (QM 6842), *P. lilacinum* (QM 4*e*), *P. notatum* (QM 944), *P. pusillum* (QM 137*g*); *Pestalotiopsis westerdijkii* (QM 381); *Pestalotia virgatula* (QM 478) *Ptychogaster rubescens* (QM 1011); *Pullularia pullulans* (QM 279*c*); *Rhizopus arrhizus* (QM 46*c*) and (QM 1032); *Schizophyllum commune* (QM 812); *Scopulariopsis brevicaulis* (QM 815 and QM 816); *Stachybotrys atra* (QM 94*d*); *Trichoderma viride* (QM 6*a*); *Bacillus megaterium* (QM B1551); *Acetobacter xylinum* (QM B1548), *Acetobacter acetigenum* (QM B1562); *Escherichia coli* (QM B1557); *Pseudomonas aeruginosa* (QM B1468); *Sporocytophaga myxococcoides* (QM B482). Low fructosanase activity, or unstable fructosanase, were found in cultures of: *Aspergillus niger* (QM 458), *A. phoenicis* (QM 1005), *A. terreus* (QM 72*f*); *Bacillus subtilis* (QM B655); *Penicillium commune* (QM 1269); and *Spicaria violacea* (QM 1031).

Active and relatively stable fructosanases were found (Table I) in culture filtrates of: *Aspergillus fumigatus* (QM 45*h*), *Fusarium moniliforme* (QM 527), *Humicola fuscoatra* (QM 34*e*), *Penicillium funiculosum* (QM 474), and *Streptomyces* sp. (QM B814). There is little relationship between the linkage present in the substrate and the enzyme found in the medium. *Fusarium moniliforme* and *Streptomyces* sp. cultures produce the 2,6-fructosanase when grown either on the 2,1- or 2,6-fructosan. Conversely, *A. fumigatus* and *Humicola fuscoatra* cultures yield 2,1-fructosanase on either substrate. As adaptive enzymes, fructosanase formation, it appears, is being stimulated by the fructose split off from the substrate, and not always by the substrate itself. Best activity was obtained in unshaken culture (though it took longer to develop). This suggests that shaking may inactivate the enzymes.

Some of the fructosanases were so unstable that they were completely inactivated at 4° C. in 2–3 days; some lost 50% of their activity in 3 weeks (e.g. the fructosanase of *A. fumigatus*); and some fructosanases were quite stable (e.g. that of *Penicillium funiculosum*). Except for *Streptomyces* sp., maximum fructosanase activity was obtained in unshaken flasks (Table I).

In the exception, peptone was used to stimulate growth. It has been observed before that protein appears to protect enzymes in shaken culture (15).

The culture filtrates contained enzymes in addition to fructosanase (Table II). Invertase activity was high in cultures of *Penicillium funiculosum* and *Fusarium moniliforme*, low in *Streptomyces* sp. and *Aspergillus fumigatus*. Amylase, maltase, and β-glucosidase were found in cell-free filtrates of *Penicillium funiculosum*; and polygalacturonidase in filtrates of *Aspergillus fumigatus*. Cellulase and xylanase were absent in all the filtrates examined.

Culture filtrates were produced that are active on either 2,1- or 2,6-fructosans or on both (Table I). Those active on only one type of linkage totally hydrolyzed that type of fructosan and yet left fructosan of another linkage type virtually intact (Table III). These fructosanase preparations were used to characterize fructosan linkage in much the same way that the structure of α- and β-glucosides is established by their susceptibility to the respective

TABLE I

EFFECT OF SUBSTRATE AND OF SHAKING ON PRODUCTION OF FRUCTOSANASES

Organism	Linkage in substrate for growth*	Maximum potency† (mg. fructose ml.$^{-1}$ hour^{-1})			
		Shaken		Unshaken	
		2,6-ase	2,1-ase	2,6-ase	2,1-ase
Fusarium moniliforme	2,6	0.05	0.01	0.60	0.05
QM 527	2,1	0.10	0.01	0.70	0.0
Streptomyces sp.	2,6	0.25	0.01	0.0	0.0
QM B 814	2,1	0.0	0.0	0.03	0.0
Penicillium funiculosum	2,6	0.26	0.84	1.1	1.22
QM 474	2,1	0.16	0.81	1.02	1.22
Aspergillus fumigatus	2,6	0.05	0.57	0.07	1.16
QM 45 H	2,1	0.0	0.10	0.19	1.22
Humicola fuscoatra	2,6	0.05	0.01	0.07	0.81
QM 34*e*	2,1	0.0	0.05	0.08	0.71

*Growth substrates: inulin = 2,1-fructosan; *Acetobacter* = 2,6-fructosan.
†See "Methods".

TABLE II

ENZYME COMPONENTS OF FRUCTOSANASE AND INVERTASE PREPARATIONS

Preparation	Activity* as hexose (mg. ml.$^{-1}$ 2 hours^{-1})						
	Fructosans				Disaccharides		
	Acetobacter	*Bacillus*	Inulin	*Aspergillus*	Sucrose	Maltose	Cellobiose
Fructosanase							
2,6 of *Streptomyces*	0.34		0.02		0.06		
2,6 of *F. moniliforme*	0.98				1.1	0	0
2,1 of *P. funiculosum*			1.21		0.76	0.40	0.70
2,1 of *A. fumigatus*			0.67		0.07	0	0
Invertase Difco (1/5)	0.09	0.08	0.03	0	1.30†	0.04	0

*Substrate 1.5 mg./ml.; pH 5.4; temperature 50° C.; time 2 hours (except *Streptomyces*, which was incubated only 1 hour).
†The maximum hydrolysis attainable. Dilution to give 50% hydrolysis of sucrose (0.65 mg. hexose ml.$^{-1}$) 1/1200.

glycosidase. Since it is known that inulin is β-2,1-linked, it is highly probable that *Aspergillus sydowi* fructosan, which behaved similarly, has the same linkage. It is also likely that *Acetobacter* fructosan has the same linkage (β-2,6) as the *Bacillus* fructosan.

Enzyme hydrolysis of the fructosans approximated 100% in all cases (Table III). The low value obtained for the *Streptomyces* sp. fructosanase is due to hydrolysis stopping almost completely at the dimer stage. The accumulation of levanbiose here is similar to the accumulation of cellobiose in the *cellulolytic* system of this organism. (We have used *Streptomyces* sp. filtrates to prepare both cellobiose and levanbiose. Each was separated from the other components of the respective hydrolyzates by the carbon column method. The yields were much better than those obtained from *acid* hydrolyzates. About 40% of the original substrate could be recovered as the difructose, levanbiose.)

When some fructosanase preparations acted on their respective substrates, no intermediates (between fructosan and fructose) were detectable chromatographically. The 2,6-fructosanases of *Fusarium moniliforme* and of *Penicillium funiculosum* and the 2,1-fructosanase of *P. funiculosum* behaved in this way. In other systems, intermediates were found (Fig. 1); these were similar to those found in acid hydrolyzates. The accumulation of intermediates is attributed to a relatively low content of oligofructosidases in the fructosanase solutions.

When culture filtrates were incubated with 7% sucrose and chromatogrammed, a slow moving fructose-containing spot (R_{fr} 0.50) and fructose were found in addition to sucrose. It is also of interest to note that commercial invertase preparations (Difco, 1/50) hydrolyzed the dimer of both the 2,1- and 2,6-series. The trimer, tetramer, and even the pentamer of both series were hydrolyzed but more slowly; yet the invertase solution had no appreciable action on either the 2,1- or the 2,6-fructosans (Table II).

Schlubach and Sinh (12) have characterized fructosans on the basis of time required to reach 50% hydrolysis in normal sulphuric acid at 20° C. In our tests, inulin, *Aspergillus* fructosan, *Bacillus* fructosan, and *Acetobacter* fructosan showed no appreciable differences; all four reached the 50% level in 157–160 minutes.

TABLE III

EXTENT OF HYDROLYSIS OF FRUCTOSANS BY ENZYMES

Fructosanase of:	Hydrolysis, %* of fructosan of:			
	Inulin	*Aspergillus*	*Acetobacter*	*Bacillus*
Penicillium funiculosum QM 474	102	113	97	98
Aspergillus fumigatus QM 45H	100	105	4	3
Humicola fuscoatra QM 34e	98	102	10	10
Fusarium moniliforme QM 527	0	0	88	82
Streptomyces sp. QM B814	0	0	50	52

*20 hours, 40° C., pH 5.4.

Acknowledgments

The cultures employed were supplied from the QM Culture Collection by Miss Dorothy Fennell. We appreciate also the information supplied to us by S. A. Barker and M. Stacey on the infrared analysis of our fructosans.

Addendum

Infrared spectra of fructosans. Report of S. A. Barker and M. Stacey (Univ. Birmingham) on our fructosans. Letter of 12/21/56.

(1) Fructosan of *Acetobacter aceligenum* (2:6 linked). "If this is indeed a polyfructose, then the peaks at 918 cm.$^{-1}$ and 800 cm.$^{-1}$ agree well with those we found at 919 cm.$^{-1}$ and 803 cm.$^{-1}$ with a levan from *Bacillus mesentericus*. The appearance of a peak at 1684 cm.$^{-1}$ would not be expected normally of a levan and suggests an impurity in the polysaccharide."

(2) Fructosan of *Aspergillus sydowi* (2:1 linked). "The peaks at 937 cm.$^{-1}$, 868 cm.$^{-1}$, and 810 cm.$^{-1}$ compare with those at 928 cm.$^{-1}$, 870 cm.$^{-1}$, and 812 cm.$^{-1}$ we found with an authentic specimen of inulin. Your extra peak at 833 cm.$^{-1}$ may be due to impurities or the presence of a proportion of α glucose residues in your polyfructosan." "You will see that these [results] confirm, in a rather nice way, your own work."

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CHARACTERISTICS OF FOUR RHIZOBIOPHAGES ACTIVE AGAINST RHIZOBIUM MELILOTI[1]

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Abstract

Four phage isolates for strains of *Rhizobium meliloti* were obtained from sewage and field soil and purified by standard techniques. Plaque sizes ranged from 1.0 to 4.0 mm. The titers of these phages remained unchanged after 24 months' storage at 0° and 4° C. None of the phages lysed all of the 38 strains of *R. meliloti* tested, although 29 of these strains were lysed by one of the phages. Of 292 other strains of rhizobia isolated from 108 species of 50 leguminous genera, one of the phages lysed six strains, another, five strains, while the other two phages did not lyse any. None of the phages lysed any of the 40 strains of *Agrobacterium* and *Chromobacterium* species tested. Inactivation rates of the four phages by their homologous antisera were linear up to 90% inactivation. Cross-reactions with heterologous antisera showed weak serological relationships between some of the phages. The latent periods of growth ranged from 80 to 90 minutes to 170–180 minutes. Burst sizes of rhizobia infected by the four phages ranged from 80 to 317 particles per bacterium after an adsorption period of 60 minutes at 30° C. The optimum pH range was 6.4–7.8. Thermal death points in broth were 51° C., 53° C., 61° C., and 70° C., respectively, for the four phages.

Introduction

Rhizobiophages are of particular interest because of the interactions possible in the phage : bacterial host : leguminous plant complex. Many of these interactions are discussed in a recent review by Allen and Allen (7).

This paper presents various descriptive features of four bacteriophages active against strains of *Rhizobium meliloti*.

Experimental Methods and Results

Isolation and Propagation

Seven source materials were tested: (*a*) nodules of alfalfa plants grown in greenhouse sand, (*b*) roots from the same plants, (*c*) the sand substrate, (*d*) soil of a 2 year alfalfa meadow, (*e*) soil from a 4 year alfalfa–ladino meadow, (*f*) raw sewage, and (*g*) certain stock cultures. Strains 103, 107-1, 127, and 135 of *R. meliloti* were used as the test organisms. A modification of Laird's M5 medium (21) consisting of K_2HPO_4, 0.5 g.; $MgSO_4.7H_2O$, 0.2 g.; NaCl, 0.1 g.; 20% yeast water, 100 ml.; and distilled water, 900 ml. was employed in the isolation procedure.

In principle, the method employed in the isolation attempts was a modification of the technique of Conn *et al.* (11). Approximately 25 g. of the source material was added to a 500 ml. flask containing 100 ml. of a 24 hour culture of a selected strain of *R. meliloti*. After an incubation period of

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24 hours at 25° C., the suspension was filtered through a Seitz filter and 1 ml. of the filtrate was added to a fresh 24 hour culture of the same strain. This procedure was repeated five times unless lysis of the culture had occurred earlier.

Following the occurrence of lysis, the lysate was filtered and plated by the double agar-layer technique (2, 19) on the aforementioned medium modified by the addition of 5 g. of mannitol, 1 g. of Difco potato extract, and 12 g. of agar per liter. This medium improved the growth of the phage : host systems and afforded distinct plaque formation and differentiation. Unless otherwise stated, this substrate, designated as Medium 2, was used throughout this study. In general, 10-fold dilutions of phage obtained by the addition of 0.1 ml. of the phage filtrate to 9.9 ml. of broth in large test tubes, 18 × 150 mm., were employed. Statistical inquiry into the accuracy of the assay technique showed that the coefficient of variation approximated nine per cent. All phage dilutions were made in either Medium 2 broth or in 2% yeast water broth.

Phages active against *R. meliloti* 103, 127, and 135 were isolated from raw sewage; others active against strains 103 and 107-1 were obtained from soil of the alfalfa–ladino meadow. Complete lysis of the homologous hosts occurred within 24–48 hours at 25–30° C. One phage isolate for each of the four strains of rhizobia was selected for study. These isolates are hereafter designated by prefixing the appropriate rhizobial strain number with the letter, P.

Plaque characteristics distinctive of each of the four phages selected for study were discernible after 48 hours' incubation at 25–30° C. Phage P103 produced clear plaques 3–4 mm. in diameter with a narrow halo of less than 1 mm. The concentric edges were sharp and distinct. Plaques produced by P107-1 were about 1.5 mm. in diameter and surrounded by a hazy halo less than 1 mm. in width. The boundaries of the clear area and the halo were usually obscure. Those formed by P127 were about 3 mm. in diameter with distinct edges and without halos; secondary growth occurred in their centers after about 5 days. The plaques formed by P135 measured about 1 mm. or less in diameter and were clear without halos but with slightly hazy edges. These descriptive features were obtained in the homologous phage–host systems and also with selected heterologous rhizobial hosts.

Phage Viability in Broth

Titers ranging between 10^7 and 10^{10} were obtained in Medium 2 broth with each of the phage isolates within an incubation period of 48 hours at 25–30° C. In anticipation of the need for ample stock suspensions of the phages throughout the study, tests were undertaken to ascertain ranges in phage viability over prolonged periods. Accordingly, 150-ml. portions of broth in 12-oz. prescription bottles were inoculated with the host organism, incubated 24 hours at 25° C., and inoculated with a 5 ml. suspension of the homologous rhizobiophage. Incubation was continued for an additional 48 hours at

25° C. After the cultures were lysed, the suspensions were filtered and the filtrate was dispensed in 10-ml. portions in sterile tubes. Respective tubes of each phage were stored at 0°, 4°, 10°, 25°, 30°, and 37° C. Plaque count assays of these filtrates were made immediately after filtration, 9 and 24 hours later, and at various intervals throughout a 94 day period.

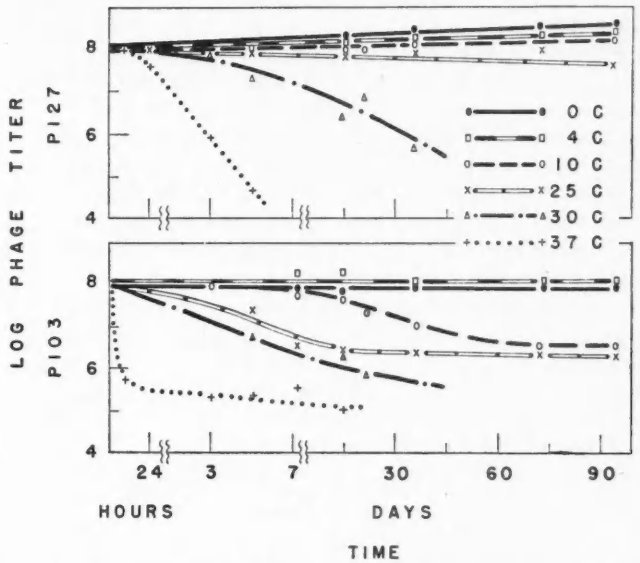


FIG. 1. Viability of phages P103 and P127 during storage at various temperatures.

The results of this experiment are shown in Fig. 1. At 0° C. and 4° C. the titer of neither phage P103 nor P127 declined within the 94 day period. At 10° C. the high titer of P127 was maintained through this period but at 25° C. a slight decrease occurred. The titer of P103 dropped early in the storage period at 10° C. and 25° C. but remained nearly constant thereafter. Similar results were obtained with this phage at the higher temperatures. The titer of P127 declined sharply at 37° C. and was lost after 5 days; similarly, a constant but less rapid decline in titer of P127 occurred at 30° C. Prolonged continuation of this experiment showed that the titers of all four phages were more or less constant over a 24 month period of storage at 4° C.

In keeping with these results, stock phage preparations were stored at 4° C. for general use. Prior to use each phage was assayed for potency.

Host Specificity Tests

Host specificity tests were undertaken for the purpose of defining further the identity of the four phages and also to ascertain the range of host infectiveness. Plates, in accordance with the double agar layer technique, were poured in which 1 ml. of a bacterial suspension was used as the inoculum in

the upper layer. Rhizobial cultures of three ages, 24, 48, or 72 hours, as determined by the growth rate of the particular strain, were used. Duplicate plates were made for each strain. After solidification of the upper layer, the plates were allowed to stand for 1 to 3 hours at 25–30° C. to permit the agar surface to dry. The plates were then subdivided into four sectors and a drop of a different phage suspension was pipetted onto each sector. Time was allowed for the agar to absorb the drops before the plates were disturbed. The occurrence of cleared areas in the film of rhizobial growth after 1–3 days depending on the growth rate of the strain was considered evidence of susceptibility.

In this manner 330 strains of rhizobia were tested in duplicate against each of the phage isolates. Thirty-eight of these were strains of *R. meliloti* isolated from 10 plant species in the alfalfa cross-inoculation group and 292 were strains from 108 species of 50 genera of other leguminous plants. In additional tests 40 strains comprising five species of the genera *Agrobacterium* and *Chromobacterium* were included.

Although it is generally agreed that host range of a phage lacks certain reliability as a taxonomic criterion because of the interdependence of the reaction on the host as well as on the phage (3, 4, 9), and also because cross-lysis may be accomplished by host range mutants of the original phage strain (5), the host specificity reactions of these phages were of particular interest. Of the 38 strains of *R. meliloti* tested, 29 were lysed by P135, 26 by P103, 6 by P127, and only 3 by P107-1. No one strain was lysed by all of the four phages. Five strains, *R. meliloti* 102, 106, 117, 142, and 143, were not lysed by any of the phages.

Of the 292 other rhizobial strains tested, 7 strains from 6 species in the genera *Lotus*, *Sesbania*, and *Astragalus* were lysed by either P103 or P135 or both. Strains designated as *Lotus corniculatus* 846, *Sesbania longifolia* 1051, *Astragalus branchocarpus* 1053, and *Astragalus pectinatus* 1060 were lysed by both phages. *Sesbania longifolia* 1052 was lysed by P135 but not by P103. Conversely, *Astragalus coulteri* 1063 and *A. wootoni* 1065 were lysed by P103 but not by P135. None of the strains in this collection was lysed by phages P127 and P107-1.

Similarly, none of the phages lysed any of the eight strains each of *Agrobacterium tumefaciens*, *A. rhizogenes*, *A. rubi*, *A. radiobacter*, and *Chromobacterium violaceum*.

Further inquiry into the host range limitations of rhizobiophages as measured by diverse rhizobial strains and closely allied bacteria invites consideration. As was judged from an extensive study of 22 phages and 35 strains of rhizobia, Conn and co-workers (11) concluded that although lytic tests probably would not separate one species of rhizobia from another, such tests were valuable as supplementary tools. A similar conclusion was reached by Bruch and Allen (8), who reported the host specificity reactions of four *Lotus* rhizobiophages against 91 *Lotus* strains, 373 strains of other rhizobia, and 40 strains of the genera *Agrobacterium* and *Chromobacterium*.

Whereas the aforementioned tests provided no information as to the virulence, or potency, of the phages for a particular strain, an approach was made by determining the relative plating efficiency (3) of these phages against the four host organisms used in the isolation procedures. The phages were diluted to a uniform concentration and 0.1-ml. suspensions were added to plating tubes containing the respective host organism. Relative plating efficiency was expressed as the ratio of plaques obtained with the heterologous host divided by the number obtained with the homologous one.

TABLE I

RELATIVE PLATING EFFICIENCY OF RHIZOBIOPHAGES ON HETEROLOGOUS HOSTS

Phage	Host	Relative plating efficiency*
P135	103	0.94
P135	127	2.02
P127	135	0.23
P103	107–1	1.14

*Plaque count of the heterologous host/plaque count of the homologous host. In each homologous series the efficiency was 1.0.

The susceptibilities of the strains of rhizobia to these phages varied within a comparatively wide range (Table I). Against *R. meliloti* 103 phage P135 had a plating efficiency of 0.94 which was not significantly different from its efficiency against its homologous host, strain 135. However, with *R. meliloti* 127 as the host, the relative efficiency was 2.02, e.g., twice as many cells of strain 127 were infected and lysed by P135 as were those of strain 135. Approximately four times as many cells of *R. meliloti* 135 were susceptible to their homologous phage than to P127. On the other hand, the susceptibility of *R. meliloti* 107-1 to P103 approximated the unity ratio obtained with the host strain 103. Thus, it appeared that the homologous rhizobial strain was not necessarily the most susceptible strain.

Serological Relationships of the Phages

Antiphage sera were produced in rabbits against each of the four phages. Filtrates of lysed bacterial cultures which titered 9×10^7, 4×10^9, 2×10^7, and 4×10^9 for P103, P107-1, P127, and P135, respectively, were used as antigens. Three milliliters of the respective antigen was injected into the marginal ear vein of rabbits on 3 successive days. Ten days later each rabbit was given an intraperitoneal injection followed by an intravenous injection on each of the 2 following days. This latter procedure was repeated 10 days later. The rabbits were trial-bled from the marginal ear vein 10 days following the last injection. When active antiserum was obtained, the animals were bled for sufficient serum which was extracted from the blood

cells in the usual manner, heated at 56° C. for 30 minutes, and stored in stoppered containers at 4° C. No preservative was added. For convenience of identification, the antisera were designated by an "S" preceding the phage symbols.

To determine the inactivation rates of each of the four phages by the anti-phage sera, 1 ml. of serum of the required dilution was added to 8 ml. of broth in a reaction tube in a water bath at 30° C. At time zero, 1 ml. of the proper phage suspension containing 1×10^7 particles per ml. was added to the reaction tube. At intervals of 2, 5, 10, 20, and 30 minutes, 0.1 ml. of the suspension was transferred and mixed with 9.9 ml. of broth. The initial 1:100 dilution was sufficient to stop the neutralizing action of the antisera. Dilutions within the range required were then made to detect phage survival by the plaque count. Plaque counts of a control series, prepared from dilutions of phage in broth, served as a basis for determining percentage survival of phage. Duplicate plates of all samples were made.

The four phages were neutralized by low concentrations of their homologous antisera (Table II). Rates of neutralization are shown in Fig. 2. The lines were plotted for best fit by the method of least squares (24) so that all points were taken into consideration. In keeping with the data on other bacterio-phages (14, 18), the rates of neutralization were characteristic of a first order reaction until 90–99% of the phage was neutralized (6, 14). The line showing neutralization of P135 was skewed inasmuch as the final value, which repre-sented greater than 90% neutralization of the phage and a reduced reaction rate, was included. Rates for P103 and P107-1 also declined slightly after 90% neutralization was reached.

TABLE II

RHIZOBIOPHAGE INACTIVATION BY ANTISERA

Phage	Antiserum	Serum dilution	Percentage of survivors after: 2 min.	5 min.	10 min.	20 min.	30 min.
P103	SP103	1 : 1,000	86.6	56.6	27.5	3.9	1.5
P103	SP107–1	1 : 10	—	79.5	72.2	90.2	89.4
P103	SP127	1 : 10	—	93.4	90.2	77.9	98.4
P103	SP135	1 : 10	—	76.2	86.1	69.7	88.5
P107–1	SP107–1	1 : 1,000	84.1	50.8	16.1	2.6	0.5
P107–1	SP103	1 : 10	—	98.9	70.1	85.1	70.2
P107–1	SP127	1 : 10	—	74.5	79.8	91.5	73.4
P107–1	SP135	1 : 10	—	100.0	77.7	83.0	95.6
P127	SP127	1 : 800	83.8	66.4	42.4	18.9	—
P127	SP103	1 : 10	—	75.0	89.6	64.6	73.0
P127	SP107–1	1 : 10	—	89.6	68.8	62.5	68.8
P127	SP135	1 : 10	—	77.1	77.1	79.2	75.0
P135	SP135	1 : 10,000	72.2	40.6	13.7	4.1	—
P135	SP103	1 : 10	—	81.4	72.9	73.8	—
P135	SP107–1	1 : 10	—	101.7	82.2	84.0	100.0
P135	SP127	1 : 10	—	92.2	94.9	96.6	—

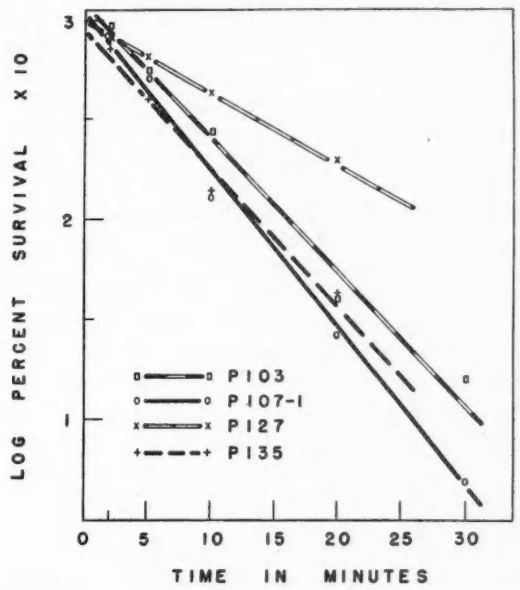


FIG. 2. Neutralization of phages by homologous antisera at 30° C.

Characteristic rates of inactivation of phage by the heterologous antisera were not obtained, although decreases in phage titer did occur. Reactions between P135 and SP107-1, P103 and SP127, and P107-1 and SP135 were within the limits of experimental error; thus, neutralization was considered not to have occurred. Significant neutralization of P127 was accomplished by all the antisera. Cross-reactions were obtained between phage and the respective heterologous antisera for P103 and P135, P103 and P107-1, and P107-1 and P127. Thus, the two phages in each of these groups appeared serologically related although the relationship was not so pronounced as has been noted for some other phage groups (5, 6).

One-step Growth Curves

Prior to the experiments on the single-step growth of the phages, growth curves of *R. meliloti* 103 and 127 were determined. For these determinations, 90-ml. portions of broth were inoculated with 10 ml. of a 72 hour culture of the test organism. Triplicate cultures for each strain were incubated at 25° C. and numbers of cells were determined hourly by the plate count method over a 12 hour period. Duration of logarithmic growth and generation times were determined from an average of the three separate sets of data.

Rhizobial cultures used in the one-step growth curve experiments were prepared by inoculating 90 ml. of broth with a 10% inoculum of a 72 hour culture of the test organism. In initial experiments these cultures were used after 6 hours' incubation at 25° C.; however, in later tests 12-hour cultures were used with comparable results.

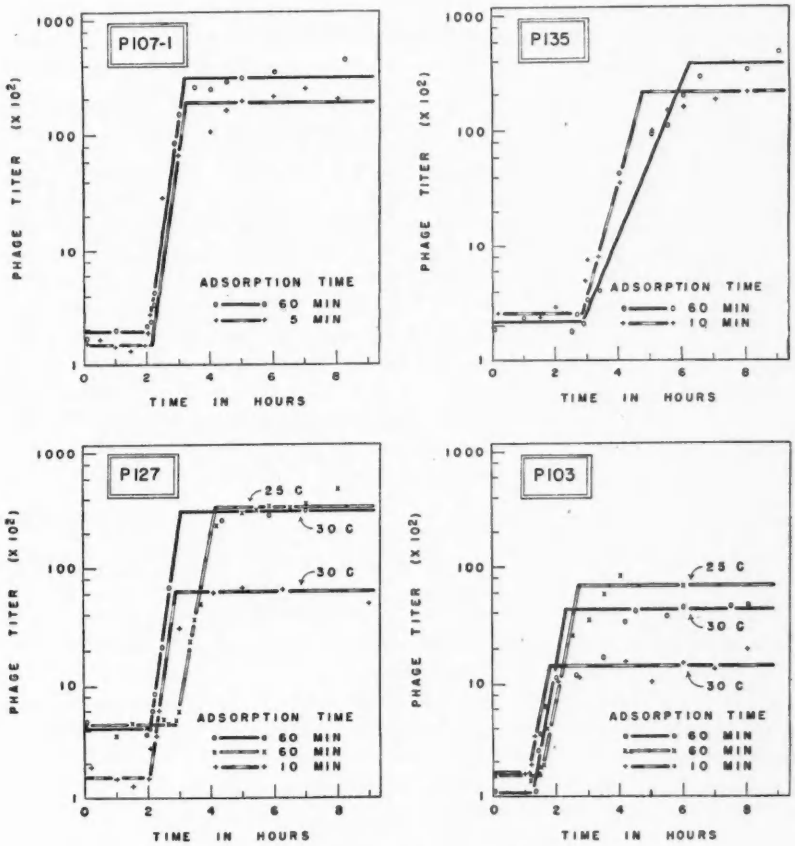
The procedure used in the one-step growth experiments was a modification of the technique of Adams (2). In order to afford a predominance of single infections, the ratio of phage to rhizobia in the adsorption tube was adjusted to provide approximately one phage particle to 10 rhizobia. Phage-specific antisera were not available at this time, whereupon adsorption was terminated by the dilution method (2). Dilution was made from the adsorption tube to the first growth tube to provide 2×10^3 phage particles per milliliter prior to the rise period. A second growth tube and usually also a third one were prepared from the first growth tube in order to provide the proper phage concentration for assay throughout the rise period. Adsorption was determined by centrifugation of the samples from the adsorption tube and assay of free phage in the supernatant. Some one-step growth experiments were conducted at 25° C.; however, the majority were conducted at 30° C. Growth curves of the four phages were determined against the homologous hosts only.

Because the best adsorption and growth of phage are obtained when the bacterial host is in the log phase of growth (13), attention was focused on the age at which the rhizobial cultures were most suitable for one-step growth experiments. Similarly, information on the generation time of the bacterial host assumed importance because adsorption time employed in the experiment should not exceed one-half the generation time of the host. The growth curves of strains 103 and 127 were very similar. The logarithmic growth of each culture extended to the 6th or 7th hour; however growth at a reduced rate continued beyond 12 hours. Generation time for these strains was 3 hours and 50 minutes. These findings were in agreement with the results of Walker *et al.* (23).

Poor adsorption of P103 and P127, which approximated only about 35%, was obtained after 1 hour in the one-step growth experiment conducted at 25° C., whereas after incubation at 30° C. for 1 hour, the adsorption percentages were 40 and 60%, respectively. In later experiments a 10 minute adsorption period was used in an attempt to refine the measurement of the latent period; during this interval adsorption of P103 and P127 was 20 to 25% and 25 to 30%, respectively.

One-step growth curves of P107-1 and P135 were determined only at 30° C., with adsorption periods of 5 and 60 minutes for P107-1 and 10 and 60 minutes for P135. Adsorption of P135 was 60–70% after 60 minutes and 20% after 10 minutes. The adsorption time of 5 minutes afforded 60% removal of the free phage particles of P107-1; after 20–30 minutes, more than 90% were adsorbed.

One-step growth curves for the four phages are shown in Figs. 3, 4, 5, and 6. The rise in titer at the end of the latent period was abrupt. Although the titer leveled off gradually as the maximum was approached, a definite plateau was obtained at the end of the one-step growth. In the comparison of individual curves, it should be noted that the initial titers in the individual experiments for a given strain were not uniform. The points for initial titer in Figs. 3–6 which represent total infective centers, infected cells, and free



FIGS. 3–6. One-step growth curves of phages P107-1, P135, P127, and P103.

phage show this variation. However, the difference did not affect expression of the final titer inasmuch as the rise in titer was plotted logarithmically.

The results of the diverse one-step growth experiments are given in Table III. Duration of the latent periods, e.g., the interval between adsorption of phage to the bacterial surface and subsequent lysis of the bacterial cells with release of newly produced phage, has been found to be a distinctive characteristic of a phage strain and independent of the bacterial host (4, 16). Duration of the latent periods ranged in length between 1 hour and 10 minutes and 3 hours. At 30° C. incubation the latent periods of P103 and P127 were shorter by 20 and 50 minutes, respectively, than at 25° C. Duration of adsorption periods had no effect on the length of the latent periods. As was indicated by the length of the latent periods, the four phages comprised

TABLE III

DATA OF ONE-STEP GROWTH EXPERIMENTS

Phage	Expt. no.	Adsorption time, min.	Temp. °C.	Adsorption, %	Latent period, min.	Burst size
P103	1	60	25	37.1	100–110	100
	2	60	25	32.8	100–110	136
	3	60	30	39.0	80– 90	92
	4	60	30	40.0	80– 90	71
	5	10	30	25.0	80– 85	35
	6	10	30	20.8	70– 80	40
P107-1	1	60	30	90.0	130–140	—
	2	60	30	97.0	125–135	169
	3	5	30	63.6	125–130	212
	4	5	30	60.3	125–130	231
P127	1	60	25	35.0	175–185	216
	2	60	30	51.0	125–135	154
	3	60	30	62.2	125–135	124
	4	60	30	60.0	125–135	141
	5	10	30	23.5	125–135	178
	6	10	30	29.4	125–135	139
P135	1	60	30	71.6	170–180	338
	2	60	30	61.4	170–180	296
	3	10	30	20.0	165–170	491
	4	10	30	18.7	170–180	441

three groups in the sense that P107-1 and P127 were closely related in contrast to P103 and P135, which appeared unrelated to each other and also to the other two phages. Latent periods of 45 and 60 minutes have been reported for phages of *Rhizobium leguminosarum* (20) and *Rhizobium meliloti* (12), respectively.

Burst sizes were calculated by the following equation:

$$\text{Burst size} = \frac{\text{final phage titer} - \text{free phage}}{\text{initial phage titer} - \text{free phage}}.$$

During the 60-minute adsorption period higher burst sizes were obtained with P103 and P127 at 25° C. than at 30° C. Burst sizes also varied according to the length of the adsorption period; thus with P107-1 and P135 the burst sizes were noticeably higher with a 10 minute adsorption period than with a 60 minute period, whereas with P103 the reverse was true. Burst size obtained with a 10 minute adsorption period for P127 in one experiment was considerably higher than that obtained with a 60 minute period, however in another experiment confirmation was not obtained. Although the results heretofore noted were consistent throughout the experiments, errors inherent in this method of determining burst size could account for the differences observed. This was particularly true in these experiments where adsorption varied widely, and free phage, as determined by centrifugation, was taken into account. Nevertheless, comparisons of burst sizes of the four phages appeared valid; accordingly, under the conditions of these experiments and

with the phage–host combinations employed, phage multiplication per bacterial cell was lowest for P103, whereas P127, P107-1, and P135 showed progressively higher burst sizes.

Effect of pH

Two series of tubes, each containing 9.9 ml. of sterile broth, were adjusted to 10 different pH values by the addition of either sodium hydroxide or hydrochloric acid. Duplicate tubes of one series were inoculated with P103 and the other with P127 to provide an initial concentration of 1×10^6 particles per ml. The tubes were stored at 25° C. Phage titers were determined at the end of 1 hour, 1 day, 1 week, and 2 weeks. Duplicate plates were poured from each sample.

Results of the periodic assays for phage survival are given in Table IV. The titers of both phages were affected similarly. Sharp decreases in titer were noted within 1 hour at pH 4.3, 4.7, 9.9, and 11.2. Between pH 5.1 and pH 8.8 the titers were constant after 1 day but after 1 week appreciable decreases were obtained. In media of levels between pH 5.7 and 7.8 the initial titers were unchanged after 1 week and declined only slightly after 2 weeks.

A similar experiment was conducted to determine phage reproduction at various pH levels. Four tubes, each containing 10 ml. of Medium 2 at eight different pH values, were inoculated with one drop of a 4-day-old broth culture of either *R. meliloti* 103 or 127 and incubated at 25° C. for 24 hours. Two tubes of each treatment were used to determine rhizobia counts; each

TABLE IV

EFFECT OF DIFFERENT pH VALUES ON PHAGE SURVIVAL

Phage	pH	1 hour	1 day	1 week	2 weeks
P127	4.1	0	0	—	—
	4.3	1×10^3	—	—	—
	4.7	2×10^4	—	—	—
	5.1	9×10^5	1×10^6	6×10^4	8×10^3
	5.7	7×10^5	9×10^5	1×10^6	1×10^6
	7.0	8×10^5	1×10^6	2×10^6	6×10^5
	7.8	1×10^6	1×10^6	9×10^5	9×10^5
	8.8	9×10^5	8×10^5	1×10^6	5×10^5
	9.9	4×10	—	—	—
	11.2	2×10	—	—	—
P103	4.1	0	0	—	—
	4.3	0	—	—	—
	4.7	8×10^3	—	—	—
	5.1	8×10^5	1×10^6	1×10^5	8×10^3
	5.7	1×10^6	2×10^6	1×10^6	6×10^5
	7.0	1×10^6	2×10^6	7×10^5	4×10^5
	7.8	5×10^5	1×10^6	1×10^6	5×10^5
	8.8	9×10^5	3×10^5	1×10^4	7×10^3
	9.9	1×10^6	—	—	—
	11.2	0	—	—	—

—, Not sampled.

TABLE V

EFFECT OF pH ON THE GROWTH OF RHIZOBIA AND RHIZOBIOPHAGE MULTIPLICATION AFTER 48 HR. INCUBATION AT 25° C.

Initial pH	Final pH		Bacterial count		Phage titer	
	103	127	103	127	P103	P127
4.1	4.4	4.4	5×10^8	3×10^8	0	0
5.1	5.4	5.5	5×10^5	3×10^6	2×10^6	6×10^5
5.7	5.9	6.0	2×10^6	2×10^6	5×10^6	3×10^7
6.0	6.1	6.4	7×10^6	2×10^6	2×10^7	3×10^8
6.4	6.6	6.8	3×10^7	7×10^6	7×10^7	3×10^9
7.0	6.9	6.8	5×10^7	5×10^7	3×10^8	2×10^9
7.8	7.7	7.8	4×10^7	5×10^7	4×10^8	2×10^9
8.8	8.6	8.7	2×10^5	5×10^6	4×10^5	9×10^5

of the other two tubes was inoculated with 0.1 ml. of the homologous phage and incubated at 25° C. for 48 hours. Phage titers were determined at the end of this period.

Close agreement between phage titer and rhizobia numbers was obtained throughout the range of pH values used (Table V). As is shown by a comparison of the two previous experiments, optimum phage proliferation was limited to a more narrow range of pH than was survival. In principle, it appeared that the major factor limiting phage growth was not pH directly but rather a manifestation of the effect of pH upon initial bacterial concentration. Relatedly, Kleczkowska (20) has shown that the depression of phage multiplication by pH was correlated with its effect upon bacterial growth. Nonetheless, the response of the phage to pH as shown in this experiment was closely related to that of phage survival.

Phage Inactivation by Heat

Rates of heat inactivation of P103 and P127 in distilled water and of all four phages in broth at pH 7.0 were determined at selected temperatures. In the experiments utilizing distilled water 8 ml. of the diluent were added to 18 × 150 mm. test tubes contained in a water bath at the desired temperature and allowed to equilibrate 10 minutes. Two milliliters of P103 and P127 each were then added to provide 2.9×10^7 and 2.5×10^7 particles per ml., respectively. Although a 2–3 degree drop in temperature occurred when this addition was made, the required temperature was recovered in 30–60 seconds; accordingly, 1 minute following the addition of phage was taken as zero time. Samples of 0.1, or 1.0 ml., were removed at intervals from the reaction tubes to broth dilution blanks kept at room temperature or into iced rhizobial suspensions. Aliquots of selected dilutions were subsequently plated. A control experiment determined survival of P103 and P127 in distilled water and in physiological saline after 2 and 10 minutes at 25° C.

At the 2 minute interval following addition of the phages to physiological saline maintained at 25° C., both P103 and P127 showed a 55% decrease in titer; no appreciable change from this level occurred at the 10 minute period.

Similarly, the loss in activity of P103 after 2 minutes in distilled water at 25° C. approximated 48%, although thereafter the titer remained constant throughout the 10 minute interval. Losses in activity of P127 at the 2 and 10 minute intervals were 43 and 52%, respectively. At temperatures of 45° C. and higher the loss in titer of P103 in distilled water ranged from 70% at 1 minute to 92% at the 10 minute interval. Similarly, at 55° C. P127 showed losses of 71% and 89% after 2 and 10 minutes, respectively. Percentage losses in activity were greater as the temperature and exposure time were increased.

In the experiments aimed at determining rates of heat inactivation in broth, the stock phage suspension was diluted to approximately 2×10^6 particles per ml. and 0.1 ml. was added to 9.9 ml. of broth in respective reaction tubes previously heated to the desired temperature in the water bath. A concentration of approximately 2×10^4 particles per ml. in the reaction tube was obtained. Since no noticeable drop in temperature occurred in the reaction tube at the time the phage suspensions were added, this was considered zero time for the experiment. Titers at the outset of the experiment were determined by diluting each phage suspension through broth dilution blanks maintained at 25° C. At designated intervals during the heating period, 0.1 ml. samples were removed from the reaction tubes and added to broth dilution blanks or to 1 ml. of a rhizobial cell suspension in plating tubes as required to obtain the necessary dilutions. All samples were plated within 1 minute after removal from the reaction tube.

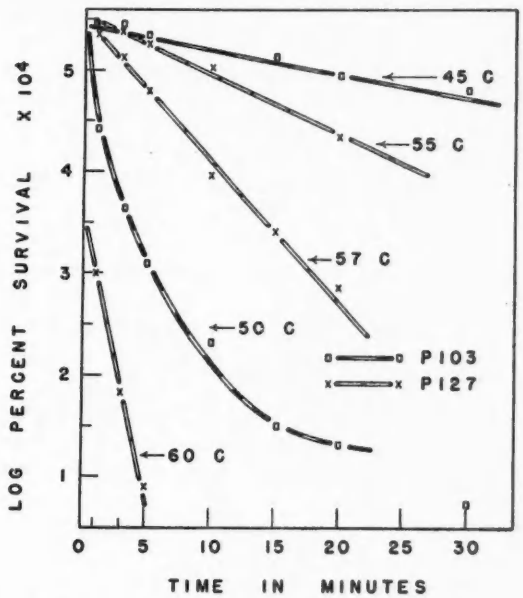


FIG. 7. Heat inactivation of phages P103 and P127 in distilled water.

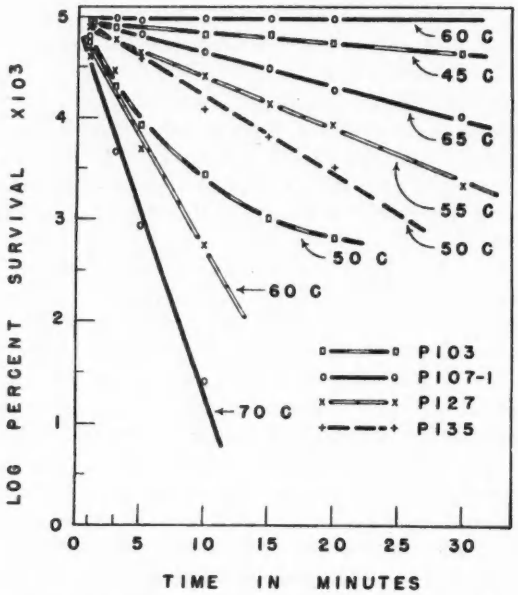


FIG. 8. Heat inactivation of phages P103, P107-1, P127, and P135 in broth at pH 7.0.

Heat-inactivation rates in distilled water and in broth are shown in Figs. 7 and 8. For phage P127 in water and for phages P107-1, P127, and P135 in broth a semilogarithmic linear relationship was found when the percentage of phage survival was plotted logarithmically against time; thus, the rate of heat inactivation of these phages evidenced a first order reaction. A variation in this pattern was shown by P103 inasmuch as the rate of inactivation decreased with time and the semilogarithmic plots resulted in a curved line (Figs. 7 and 8). At 45° C. the changes in phage titer were small and the sensitivity of the phage titration was not sufficient to manifest a curved line.

Although first order reaction rates indicative of the homogeneity of heat labile material are more commonly found (1, 10, 17), inactivation rates similar to the one obtained with P103 have been noted with other phages (15, 22). The possibility that the suspension of P103 consisted of a heterogeneity of heat labile material was not fully investigated.

In Table VI are listed rate constants calculated for the phages at various temperatures. The rate velocity constant, K, was determined by the formula: $K = 2.303 \times$ slope. Determination of the slope was by the method of least squares (24):

$$b = \frac{Sxy - (SxSy/n)}{Sx^2 - (Sx)^2/n}$$

where b = slope, x = any value on the x axis, y = a corresponding value on the y axis, xy = the product of a value of x and the corresponding value

TABLE VI

RATE CONSTANTS (K) OF THERMAL INACTIVATION

Temperature, °C.	Phage	K	
		Water	Broth
45	P103	0.0544	0.0246
50	P135		0.1757
55	P127	0.1396	0.1227
57	P127	0.3123	
60	P127	1.2408	0.4995
60	P107-1		0.0034
65	P107-1		0.0755
70	P107-1		0.8362

of y, n = the number of pairs of x and y, and S = the summation. The calculated slopes correspond to the lines plotted in Figs. 7 and 8.

As was to be expected, the K-values for heat inactivation of the phages in water were greater than those obtained in broth (Table VI). This was likely a manifestation of the protective action of the broth against heat penetration. Doubt exists that the rapid drop in phage titer which occurred during the first minute following addition of phage to water contributed noticeably to the differentials in K-values. Had this been true and, moreover, had this initial decrease in titer been projected measurably beyond the interval of 1 minute, the rate of heat inactivation of P127 in distilled water would likely have decreased throughout the 1–10 minute sampling interval; in consequence, a straight line rate function would not have been obtained.

Thermal death points for each of the four phages were also determined. Reaction tubes were filled with broth and prepared in the manner previously described. The initial phage concentration in each reaction tube was 2×10^4 particles per ml. At the end of a 10 minute period following addition of 0.1 ml. of the phage to the reaction tube, a 0.1 ml. sample was removed and placed in each of two tubes containing 5 ml. of a 24 hour broth culture of the homologous bacterial host. Lysis of the culture after sufficient incubation was considered evidence that the phage had survived.

The thermal death points of the phages are shown in Table VII. The data were based upon lysis or non-lysis of rhizobia cultures inoculated with samples of phage suspension from the reaction tube. The demarcation between lysis and non-lysis was sharp and the temperature that inactivated phage sufficiently to prevent visible lysis was easily determined. Thus, the thermal death point for P103 after 10 minutes was 51° C. and that for P135 was slightly higher at 53° C. The thermal death points for P107-1 and P127 were 70° C. and 61° C., respectively. The order of sensitivity to heat found in this experiment was in agreement with that found in the rate studies.

TABLE VII

THERMAL INACTIVATION AFTER EXPOSURE TO HEAT FOR 10 MINUTES

Experiment		Phage											
		P103			P107-1			P127			P135		
		Temperature, ° C.											
		49	50	51	68	69	70	60	61	62	51	52	53
I	1	+	+	−	+	+	−	+	−	−	+	+	−
	2	+	+	−	+	+	−	+	−	−	+	+	−
II	1	+	+	−	+	−	−	+	+	−	+	+	−
	2	+	+	−	+	+	−	+	−	−	+	+	−
III	1	+	+	−	+	+	−	+	−	−	+	+	−
	2	+	−	−	+	+	−	+	−	−	+	+	−

+ = Lysis. − = No lysis.

Summary

In the present study four rhizobiophages, P103, P107-1, P127, and P135, as designated by the strains used in the isolation techniques are described. A summary of the major descriptive features obtained is given in Table VIII.

Serologically the phages were weakly related, however relatedness of P103 to P135 and of P107-1 to P127 was indicated. On the basis of latent period determinations P107-1 and P127 were closely related, whereas P103 and P135 were unrelated to each other and also to the other two phages. P103 and P135 were closely related in their sensitivity to heat; neither P107-1 nor P127 was related to any one of the other three phages. Failure to obtain a characteristic rate of inactivation for P103 distinguished it in this regard. On the basis of these criteria it was concluded that these four phages comprised two general groups, P103 and P135, and P107-1 and P127. Despite the conventional opinion (3, 4, 9) that host range is not necessarily a valid criterion of phage characterization, the magnitude of the host specificity reactions obtained conformed with these aforementioned groupings.

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TABLE VIII

SUMMARY OF THE MAJOR DESCRIPTIVE CHARACTERISTICS OF FOUR ALFALFA RHIZOBIOPHAGES

Phage	Source	Plaque description	Host range Strains lysed		Latent period, min.	Burst size	Thermal death point
			R. meliloti (38 tested)	Other rhizobia (292 tested)			
P103	Field soil	Clear, narrow distinct halo, 3–4 mm. diam.	26	6	80– 90	71– 92	51° C.
P107-1	Field soil	Clear, hazy halo, 1.5 mm. diam.	3	0	125–135	169	70° C.
P127	Sewage	Clear followed by secondary growth, no halo, 3 mm. diam.	6	0	125–135	124–154	61° C.
P135	Sewage	Clear, no halo, 1 mm. diam.	29	5	170–180	296–338	53° C.

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NOTES

THREE MORE VIRUSES BORNE AT THE STYLET TIPS OF THE APHID MYZUS PERSICAE (SULZ.)

R. H. E. BRADLEY[1] AND R. Y. GANONG[1]

This note reports that cucumber mosaic, henbane mosaic, and tobacco severe etch viruses are borne at the stylet tips of the aphid vector *Myzus persicae* (Sulz.). This was shown in the same way that it was for potato virus Y (1). Dr. B. Kassanis, Rothamsted Experimental Station, supplied the viruses, except the Canadian strain of cucumber mosaic virus, which was supplied by Dr. C. D. McKeen, Science Service Laboratory, Harrow, Ontario.

In all the experiments, wingless adult aphids transmitted each virus from infected White Burley tobacco plants (*Nicotiana tabacum* L.) to healthy young plants of the same species. To ensure that most aphids inserted more than the tips of their stylets into infected plants, single aphids were observed on infected plants for about five minutes, and used only if they probed continuously for over a minute. Examination of aphids anesthetized in the feeding position after a minute's probing showed that they usually had their stylets inserted over 30 microns. Aphids that had probed over a minute were removed and allowed 30 seconds to extend the labium over the stylets. Then the aphid was anesthetized with carbon dioxide (2), the tip 15 microns of its stylets bared by manipulating the labium with a capillary tube (2), and the aphid exposed to ultraviolet radiations for 30 seconds (1). As controls, aphids were treated in the same way except that, after the stylets were bared, the labium was manipulated a second time so that it covered the stylet tips and thereby shielded them from the radiations (2). After irradiation, each aphid was tested for infectivity by being placed overnight on a young tobacco plant. Five aphids were tested for each of the two treatments, and the experiment repeated eight times—a separate series for each virus. Shown below are the numbers of infective aphids out of 40 tested:

	Irradiated with stylets bared 15 μ	Irradiated without stylets bared
Cucumber mosaic virus		
Canadian strain	0	9
Rothamsted strain	0	12
Henbane mosaic virus	2	15
Tobacco severe etch virus	0	10

Most aphids were made noninfective when they were irradiated with the tip 15 microns of their stylets bared, but not when the tips of the stylets were shielded from the radiations. Since the results are very similar to those

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obtained with potato virus Y (1), it seems that these viruses are also borne at the stylet tips. And even though more than the tip 15 microns of the stylets were inserted into infected plants, only the tip 15 microns or less appears to carry virus that is transmitted.

Besides these viruses, many others are transmitted when their aphid vectors make brief probes with their mouth parts. Whether they are also borne at the stylet tips could be shown by a simple experiment like that described above. Presumably most of them are; but there may be exceptions, and these would be the more interesting to find.

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ON THE USE OF 'M.I.F.' FIXATIVE–STAIN IN THE DIAGNOSIS OF TRICHOMONIASIS IN HUMANS

L. P. E. CHOQUETTE AND J.-M. DESRANLEAU

Trichomonas vaginalis in humans is widely distributed and, while its role has not been definitely established, many physicians attribute to it a certain degree of pathogenicity. The diagnosis of trichomoniasis in the human female is based on the recovery of trichomonads in the urine and vaginal discharge or mucosal scrapings of the vagina.

Diagnosis of the condition presents no difficulty when the patient is confined to the hospital or when the physician is able to forward to the laboratory in a relatively short time suitable material for diagnostic purposes. However, this is not always the case, and often material collected by the physician will reach the laboratory after several days' delay and often after being kept under conditions unfavorable to the parasite's survival; such material is far from being satisfactory. Temperature requirements rule out the use of culture media. It is evident that a medium that would preserve material in a suitable state for examination in spite of delays and temperature conditions would be of some value.

The 'M.I.F.' (merthiolate-iodine-formaldehyde) fixative–stain as described by Sapero and Lawless (1) for the identification of intestinal protozoa proved to be such a medium, particularly when the material to be examined consists

of vaginal discharge or mucosal scrapings of the vagina. The stock solution consists of distilled water, 250 cc.; tincture of merthiolate No. 99 Lilly (1:1000), 200 cc.; formaldehyde U.S.P., 25 cc.; and glycerol, 5 cc.

The procedure followed by the writers is very simple. A vaginal swab from a female patient suspected of harboring the parasite is dropped in a screw-top 25 cc. capacity test tube containing 5 cc. of 'M.I.F.' fixative–stain. In the laboratory the test tube is shaken for a few seconds and its liquid contents transferred to a centrifuge tube, which is then revolved in a centrifuge at a speed of about 2000 r.p.m. for 1 to 2 minutes. The supernatant fluid is removed with a pipette and a drop of the sediment transferred to a slide. A smaller drop of freshly prepared Lugol's solution is added and the preparation is thoroughly mixed. The preparation is then mounted with a cover glass and examined. In such preparation the trichomonads are easily recognized by their light staining in comparison with the dark staining of polymorphonuclear leucocytes and especially by their four anterior free flagella which are easily seen; the nucleus and axostyle will be seen upon proper focusing but not the undulating membrane. Material preserved in this fixative–stain can certainly be kept for a long period of time with the trichomonads retaining their diagnostic features.

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